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(57) Abstract

The subject invention provides recombinant nucleic acid molecules which encode a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera, and the proteins encoded thereby. The subject invention further provides host vector systems for the production of a BPI variant, LBP variant, LBP-BPI chimera, BPI-IgG chimera, LBP-IgG chimera, or LBP-BPI-IgG chimera, and methods of using same for producing said proteins. The subject invention provides a pharmaceutical composition, which comprises a therapeutically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera, and a pharmaceutically acceptable carrier, and the method of using same to treat a subject suffering from an endotoxin-related disorder. Finally, the subject invention provides a method of preventing an endotoxin-related disorder in a subject, which comprises administering to the subject a prophylactically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera.

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RECOMBINANT BPI-BASED AND LBP-BASED PROTEINS, NUCLEIC ACID MOLECULES ENCODING SAME, METHODS OF PRODUCING SAME, AND USES THEREOF

This application is a continuation-in-part of U.S. Serial No. 08/165,717, filed December 10, 1993, which is a continuation-in-part of (a) U.S. Serial No. 08/056,292, filed April 30, 1993, which is a continuation-in-part of U.S. Serial No. 07/567,016, filed August 13, 1990, and of PCT International Application No. PCT/US91/05758, filed August 13, 1991, and (b) PCT International Application No. PCT/US92/08234, filed September 28, 1992. PCT International Application No. PCT/US92/08234 designates the United States of America as a continuation-in-part of U.S. Serial No. 07/766,566, filed September 27, 1991, which is a continuation-in-part of U.S. Serial No. 07/681,551, filed April 5, 1991. PCT International Application No. PCT/US91/05758 designates the United States of America as a continuation-in-part of U.S. Serial No. 07/567,016, filed August 13, 1990, and 07/681,551, filed April 5, 1991, which is a continuation-in-part of U.S. Serial No. 07/567,016, filed August 13, 1990, which is a continuation-in-part of U.S. Serial No. 07/468,696, filed January 22, 1990, which is a continuation-in-part of U.S. Serial No. 07/310,842, filed February 14, 1989, the contents of all of which are hereby incorporated by reference.

Background of the Invention

Throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

Gram-negative infections are a major cause of morbidity and

mortality, especially in hospitalized and immunocompromised patients. [Duma, R.J., Am. J. of Med., 78 (Suppl. 6A):154-164 (1985); and Kreger, B.E., D.E. Craven and W.R. McCabe, Am. J. Med., 68:344-355 (1980)]. Although available antibiotics are generally effective in containing Gram-negative infections, they do not neutralize the patho-physiological effects associated with heat stable bacterial toxins (called endotoxins or lipopolysaccharides (LPS)) which are released from the outer membrane of Gram-negative bacteria upon lysis [Shenep, J.L. and K.A. Morgan, J. Infect. Dis., 150(3):380-388 (1984)]. Endotoxin is a potent stimulator of the inflammatory response. Endotoxemia occurs when endotoxin enters the bloodstream resulting in a dramatic systemic inflammatory response.

Many detrimental effects of LPS in vivo result from soluble mediators released by inflammatory cells. [Morrison, D.C. and R.J. Ulevitch, Am. J. Pathol, 93(2):527-617 (1978)]. Monocytes and neutrophils play a key role in this process. These cells ingest and kill microorganisms intracellularly and also respond to endotoxin in vivo by releasing soluble proteins with microbicidal, proteolytic, opsonic, pyrogenic, complement-activating and tissue-damaging effects. Tumor necrosis factor (TNF), a cytokine released by endotoxin-stimulated monocytes, mimics some of the toxic effects of endotoxin in vivo. Injecting animals with TNF causes fever, shock, and alterations in glucose metabolism. TNF is also a potent stimulator of neutrophils. Other cytokines such as IL-1, IL-6, and IL-8 also mediate many of the patho-physiologic effects of LPS, as well as other pathways involving endothelial cell activation by tissue factor, kininogen, nitric oxide and complement.

The presence of endotoxin and the resulting inflammatory response may result, for example, in disseminated intra-

vascular coagulation (DIC), adult respiratory distress syndrome (ARDS), cardiac dysfunction, organ failure, liver failure (hepatobiliary dysfunction), brain failure (CNS dysfunction), renal failure, multi-organ failure and shock.

Diseases associated with endotoxemia include, by way of example, the systemic inflammatory response syndrome (SIRS), sepsis syndrome, septic shock, bacterial meningitis, neonatal sepsis, cystic fibrosis, inflammatory bowel disease and liver cirrhosis, gram-negative pneumonia, gram-negative abdominal abscess, hemorrhagic shock and disseminated intravascular coagulation. Subjects that are leukopenic or neutropenic, including subjects treated with chemotherapy or immunocompromised subjects (for example with AIDS), are particularly susceptible to bacterial infection and the subsequent effects of endotoxin. Endotoxin-associated disorders can be present whenever there is a gram-negative infection. Endotoxin-associated disorders can also be present (a) when there is ischemia of the gastrointestinal tract, which ischemia may be present following hemorrhagic shock or during certain surgical procedures, or (b) when systemic or local inflammation causes increased permeability of the gut to endotoxin or gram-negative organisms.

Current methods for treating Gram-negative infections use antibiotics and supportive care. Despite successful antimicrobial therapy, morbidity and mortality associated with endotoxemia remain high. Antibiotics are not effective in neutralizing the toxic effects of LPS. Therefore, the need arises for a therapy with direct endotoxin-neutralizing activity.

Polymyxin B (PMB) is a basic polypeptide antibiotic which has been shown to bind to, and structurally disrupt, the most toxic and biologically active component of endotoxin--

Lipid A. PMB has been shown to inhibit endotoxin activation of neutrophil granule release in vitro and is a potential therapeutic agent for Gram-negative infections. However, because of its systemic toxicity, this antibiotic has limited therapeutic use except as a topical agent.

Combination therapy using antibiotics and high doses of methylprednisolone sodium succinate (MPSS) has been shown to prevent death in an experimental model of Gram-negative sepsis using dogs. However, a study using MPSS with antibiotics in a multi-center, double blind, placebo-controlled clinical study in 223 patients showing clinical signs of systemic sepsis showed that the mortality rates were not significantly different between the treatment and placebo groups [Bone, R.C., et al., N. Engl. J. of Med. 317:653 (1987)].

A relatively new approach to the treatment of endotoxemia is passive immunization with endotoxin-neutralizing antibodies. Hyperimmune human immunoglobulin against E. coli J5 has been shown to reduce mortality by 50% in patients with Gram-negative bacteremia and shock. Other groups have proposed using mouse, chimeric, and human monoclonal antibodies directed to endotoxin. However, these antibodies do not neutralize endotoxin.

Another mode of treating endotoxemia involves the use of cytokine blockers, such as IL-1 receptor antagonist and anti-TNF antibodies, as well as the soluble forms of the IL-1 and TNF receptors. However, a cytokine blocker can only block the cytokine(s) for which it is specific, and cannot block other cytokines. Furthermore, blocking cytokines may have other deleterious effects.

Two soluble endotoxin-binding proteins have now been

identified which play a role in the physiological response to endotoxin. One, lipopolysaccharide binding protein (LBP), is a soluble receptor found in serum which mediates endotoxin activation of cells. The second, bactericidal/ permeability-increasing protein (BPI), binds and neutralizes endotoxin, preventing inflammatory cell activation. These two natural binding proteins play opposing roles in determining the fate of endotoxin and how the body responds to a localized or systemic Gram-negative infection.

In the 1980's, Ulevitch and coworkers reported the isolation of a protein from rabbit acute phase serum which binds LPS with a high affinity [Tobias, P.S., Soldau, K. and Ulevitch, R.J. (1986) J. Exp. Med. 164:777-793]. They called this protein lipopolysaccharide binding protein (LBP). LBP was subsequently shown to stimulate opsonization of LPS-coated particles by monocytes [Wright, S.D., Tobias, P.S., Ulevitch, R.J. and Ramos, R.A. (1989) J. Exp. Med. 170:1231-1241]. LBP was further shown to bind to the lipid A moiety of endotoxin, which binding accounts for much of the biological activity of endotoxin [Tobias, P.S., Soldau, K. and Ulevitch, R.J. (1989) J. Biol. Chem. 264:10867-10871].

BPI is a neutrophil granule protein first discovered in 1975 [Weiss, J., R.C. Eranson, S. Becherdite, K. Schmeidler, and P. Elsbach, J. Clin. Invest. 55:33 (1975)]. BPI was obtained in highly purified form from human neutrophils in 1978 and was shown to increase membrane permeability and to have bactericidal activity against Gram-negative bacteria when assayed in phosphate buffered saline <u>in vitro</u> [Weiss, J., et al., J. Biol. Chem, 253:2664-2672 (1978)]. Weiss, et al. showed that BPI increases phospholipase A2 activity, suggesting a proinflammatory activity for BPI in addition to its <u>in vitro</u> bactericidal activity [Weiss et al., J. Biol. Chem. 254:11010-11014 (1979)].

Rabbit BPI was purified in 1979 [Elsbach et al., J. Biol. Chem. 254:11000-11009] and shown to have bactericidal and permeability increasing properties identical to those of BPI from humans. Rabbit BPI was thus shown to be a further source of material for study. Both rabbit and human BPI were shown to be effective against a variety of Gram-negative bacteria in vitro, including Kl-encapsulated E. coli [Weiss et al., Infection and Immunity 38:1149-1153 (1982)].

In 1984, a protein with properties similar to BPI was isolated from human neutrophils and designated cationic antimicrobial protein 57 (CAP 57) [Shafer, W.M., C.E. Martin and J.K. Spitznagel, Infect. Immun. 45:29 (1984)]. In 1986, Hovde and Gray reported a bactericidal glycoprotein with properties virtually identical to those of BPI [Hovde and Gray, Infection and Immunity 54(1):142-148 (1986)].

A role for lipopolysaccharide in the in vitro bactericidal action of BPI was proposed in 1984 by Weiss et al. [J. Immunol. 132(6):3109-3115 (1984)]. Weiss, et al. demonstrated that BPI binds to the outer membrane of Gram-negative bacteria, causes the extracellular release of LPS, and selectively stimulates LPS biosynthesis.

In 1985, Ooi et al. reported that BPI retains its in vitro bactericidal activity after cleavage with neutrophil proteases, suggesting that fragments of the molecule retain activity [Ooi and Elsbach, Clinical Research 33(2):567A (1985)]. All of the in vitro bactericidal and permeability increasing activities of BPI are present in the N-terminal 25 kD fragment of the protein [Ooi, C.E., et al., J. Biol. Chem. 262:14891 (1987)]. BPI binding to Gram-negative bacteria was reported originally to disrupt LPS structure, alter microbial permeability to small hydrophobic molecules

and cause cell death [Weiss, et al. (1978)].

Molecular Structures of BPI and LBP

BPI shares amino acid sequence homology and immunocrossreactivity with LBP [Tobias et al., J. Biol. Chem. 263:13479-13481 (1988)], and the genes encoding both BPI and LBP have been cloned [Gray, P.W., Flaggs, G., Leong, S.R., Gumina, R.J., Weiss, Ooi, C.E. and Elsbach, P. (1989) J. Biol. Chem. 264:9505-9509]. Both genes code for hydrophobic leader sequences and polypeptides having 44% amino acid sequence identity. LBP was reported by Schumann et al. to contain four cysteine residues and five potential glycosylation sites, whereas BPI contains three cysteine residues and only two glycosylation sites. It should be noted that the cDNA sequence and protein sequence of LBP used herein are distinct from those published by Schumann et al., including significant differences such as the absence of a cysteine and an insertion of four amino acids. As used herein, LBP means a protein having the sequence shown for human LBP in Figure 5. BPI can be described as having two distinct domains, an N-terminal domain, and a C-terminal domain, which domains are separated by a proline-rich hinge region. The N-terminal domain of the LBP molecule has been shown to contain the bactericidal and LPS-binding domain of BPI [Ooi and Elsbach, Clinical Research 33(2):567A (1985) and Ooi, C.E., et al., J. Biol. Chem. 262:14891 (1987)]. The C-terminal domain of BPI has been reported to have modest LPS-binding activity. The C-terminal domain of LBP is thought to be involved in the binding and activation of monocytes. The N- and C-terminal domains of BPI have a striking charge asymmetry that is not shared by LBP. The N-terminal domain of BPI is extremely rich in positively charged lysine residues, and this charge imparts a predicted pI > 10 to the full-length molecule, whereas the C-terminal domain is slightly negatively charged. The bactericidal

activity of BPI may result from its cationicity. LBP is largely neutral, has no skewed charge distribution, and is not bactericidal [Tobias, P.S., Mathison, J.C. and Ulevitch, R.J. (1988) J. Biol. Chem. 263:13479-13481]. The putative functions of the N- and C-terminal domains of BPI and LBP are illustrated in Figure 2. Table 1 provides a comparison of BPI and LBP structure and function.

Therapeutic Applications of BPI and LBP

Therapeutic intervention to block the inflammatory effects of LPS can ameliorate the morbidity and mortality associated with endotoxemia and septic shock. Unfortunately, native BPI has an extremely short half-life in the human blood-stream which limits its use in therapy. Native LBP has a longer half-life but elicits in the presence of endotoxin a brisk monocyte reaction which if excessive can cause the release of deleterious quantities of cytokines. An ideal candidate would have a longer half-life and effective endotoxin binding/inactivation without monocyte stimulation.

Table 1

Comparison of BPI and LBP Structure and Function

	BPI	LBP
Synthesis		
Site of synthesis	Neutrophil	Liver
Blood concentration	1-10 ng/ml	1-10 μg/ml
Structure		
Molecular Mass	55 kD	60 kD
Glycosylation sites	2	5
Cysteine	3	4(3)*
Effects on LPS mediated:		
Neutrophil activation	Inhibits	Stimulates
Monocyte activation	Inhibits	Stimulates
TNF release	Inhibits	Stimulates
IL-1 release	Inhibits	Stimulates
IL-6 release	Inhibits	Stimulates

*Four cysteines are reported by Schumann et al. [Science 249:1429-1431 (1990)] but the inventors have only found three (see Figure 1).

Summary of the Invention

The subject invention provides a recombinant nucleic acid molecule which encodes a BPI variant. The subject invention also provides the BPI variant encoded by the recombinant nucleic acid molecule of the subject invention.

The subject invention further provides a vector comprising the recombinant cDNA molecule of the subject invention. The subject invention further provides a host vector system for the production of a BPI variant, which comprises the vector of the subject invention in a suitable host.

The subject invention further provides a method for producing a BPI variant, which comprises growing the host vector system of the subject invention under conditions permitting the production of the BPI variant and recovering the BPI variant produced thereby.

The subject invention provides a recombinant nucleic acid molecule which encodes an LBP variant. The subject invention also provides the LBP variant encoded by the recombinant nucleic acid molecule of the subject invention.

The subject invention further provides a vector comprising the recombinant cDNA molecule of the subject invention. The subject invention further provides a host vector system for the production of an LBP variant, which comprises the vector of the subject invention in a suitable host.

The subject invention further provides a method for producing an LBP variant, which comprises growing the host vector system of the subject invention under conditions permitting the production of the LBP variant and recovering the LBP variant produced thereby.

The subject invention provides a recombinant nucleic acid molecule which encodes an LBP-BPI chimera. The subject invention also provides the LBP-BPI chimera encoded by the recombinant nucleic acid molecule of the subject invention.

The subject invention further provides a vector comprising the recombinant cDNA molecule of the subject invention. The subject invention further provides a host vector system for the production of an LBP-BPI chimera, which comprises the vector of the subject invention in a suitable host.

The subject invention further provides a method for producing an LBP-BPI chimera, which comprises growing the host vector system of the subject invention under conditions permitting the production of the LBP-BPI chimera and recovering the LBP-BPI chimera produced thereby.

The subject invention provides a recombinant nucleic acid molecule which encodes a BPI-IgG chimera. The subject invention also provides the BPI-IgG chimera encoded by the recombinant nucleic acid molecule of the subject invention.

The subject invention further provides a vector comprising the recombinant cDNA molecule of the subject invention. The subject invention further provides a host vector system for the production of a BPI-IgG chimera, which comprises the vector of the subject invention in a suitable host.

The subject invention further provides a method for producing a BPI-IgG chimera, which comprises growing the host vector system of the subject invention under conditions permitting the production of the BPI-IgG chimera and recovering the BPI-IgG chimera produced thereby.

The subject invention provides a recombinant nucleic acid

molecule which encodes an LBP-IgG chimera. The subject invention also provides the LBP-IgG chimera encoded by the recombinant nucleic acid molecule of the subject invention.

The subject invention further provides a vector comprising the recombinant cDNA molecule of the subject invention. The subject invention further provides a host vector system for the production of an LBP-IgG chimera, which comprises the vector of the subject invention in a suitable host.

The subject invention further provides a method for producing an LBP-IgG chimera, which comprises growing the host vector system of the subject invention under conditions permitting the production of the LBP-IgG chimera and recovering the LBP-IgG chimera produced thereby.

The subject invention provides a recombinant nucleic acid molecule which encodes an LBP-BPI-IgG chimera. The subject invention also provides the LBP-BPI-IgG chimera encoded by the recombinant nucleic acid molecule of the subject invention.

The subject invention further provides a vector comprising the recombinant nucleic acid molecule of the subject invention. The subject invention further provides a host vector system for the production of an LBP-BPI-IgG chimera, which comprises the vector of the subject invention in a suitable host.

The subject invention further provides a method for producing an LBP-BPI-IgG chimera, which comprises growing the host vector system of the subject invention under conditions permitting the production of the LBP-BPI-IgG chimera and recovering the LBP-BPI-IgG chimera produced thereby.

The subject invention provides a pharmaceutical composition, which comprises a therapeutically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera, and a pharmaceutically acceptable carrier.

The subject invention further provides a method of treating a subject suffering from an endotoxin-related disorder, which comprises administering to the subject a dose of the pharmaceutical composition of the subject invention effective to bind to LPS and thereby inhibit LPS-mediated stimulation of neutrophils and mononuclear cells, so as to thereby treat the subject.

Finally, the subject invention provides a method of preventing an endotoxin-related disorder in a subject, which comprises administering to the subject a prophylactically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera, so as to thereby prevent the endotoxin-related disorder in the subject.

Brief Description of the Figures

Figures 1A and 1B

Differences between LBP sequence as used herein (LBP-b) and LBP sequence as published by Schumann, et al. (LBP-a).

Figure 2 Model for the interaction of BPI and LBP with LPS and monocytes. LBP binds to LPS to form the LPB-LPS complex which then binds CD14 and activates monocytes to produce inflammatory cytokines. BPI binds to LPS but the BPI-LPS complex does not bind CD14 or activate monocytes.

Figures 3A and 3B

BPI nucleotide and amino acid sequences.

Figures 4A and 4B

LBP nucleotide and amino acid sequences.

Figures 5A and 5B

Aligned amino acid sequences of BPI and LBP proteins from various species.

Figure 6 Amino acid sequence of NCY118 protein.

Figures 7A and 7B

Human IgG-1 amino acid and nucleotide sequences.

Figure 8 Effects of BPI, NCY102, NCY103 and NCY104 on $^{\text{biotinylated}}$BPI binding to LPS.

Figure 9 Effects of BPI, NCY102, NCY103, NCY104 and NCY105 protein on LPS activity in the chromogenic LAL assay.

Figure 10 FITC-LPS binding to monocytes in the presence of BPI and NCY103.

Figure 11 Effects of BPI, NCY102, NCY103 and NCY104, on TNF release by LPS in whole blood.

Figure 12 Clearance of BPI, NCY102, NCY103 and NCY104 from mouse serum after intravenous injection.

Figure 13 Comparison of the efficacy of BPI and NCY103 given before endotoxin challenge.

Figure 14 Effects of BPI, NCY103, NCY118, NCY114, NCY115, and NCY117 on biotinylated BPI binding to LPS.

Figure 15 Effects of BPI, LBP, NCY103 and NCY104 on FITC-labeled LPS binding to human peripheral blood monocytes in the presence of 10% autologous serum (panel A) and in the absence of serum and presence of 0.5% human serum albumin (panel B).

Figure 16 Comparison of the effects of LBP vs. NCY103, NCY104, NCY117 and PLL (poly-L-lysine) on the stimulation of TNFα release by phorbol ester-induced THP-1 cells in the absence of serum.

Figure 17 LPS-mediated TNF production in THP-1 cells cultured without serum.

Figures 18A, 18B, 18C, 18D, 18E and 18F

Clearance of: BPI, LBP, NCY103, NCY104 and NCY118 (panel A); BPI, NCY114, NCY115 and NCY139 (panel B); BPI, LBP, NCY117 and NCY118 (panel C); BPI, LBP and NCY144 (assayed for both Fc and BPI) in CD-1 mice (panel D); LBP, NCY116, NCY117, NCY118 (panel E); NCY102, NCY103, NCY115, NCY135, and NCY134 (panel F); NCY102, NCY141, NCY142, NCY143, and BPI (panel G); and BPI, NCY115, and NCY114 (panel H).

Figure 19 Western blot of BPI and NCY118 produced in Pichia pastoris.

Figure 20 Effects of BPI and NCY103 on endotoxin activation of monocytes.

Detailed Description of the Invention

Toward the goal of ameliorating the morbidity and mortality associated with endotoxemia and septic shock, the subject invention provides BPI and LBP variants, BPI-LBP chimeras, and BPI-IgG and LBP-IgG chimeras having biological properties distinct from and advantageous to either native BPI or native LBP. The subject invention also provides therapeutic and prophylactic uses for these molecules.

Specifically, the subject invention provides a recombinant nucleic acid molecule which encodes a BPI variant. In one embodiment, the recombinant nucleic acid molecule is a DNA molecule. In the preferred embodiment, the DNA molecule is a cDNA molecule.

As used herein, BPI or bactericidal permeability increasing protein means a protein having the amino acid sequence shown for human BPI in Figure 5. The BPI nucleotide and amino acid sequences are shown in Figure 3.

As used herein, a BPI variant means a protein comprising a portion of BPI, which protein is capable of (a) binding to LPS, (b) competing with BPI or LBP for binding to LPS, and (c) inhibiting the LPS-mediated production of TNFα by human monocytes. For example, a BPI variant may comprise a fragment of BPI, a point mutant of BPI, a deletion mutant of BPI, or both a point and deletion mutant of BPI.

As used herein, LPS means lipopolysaccharide, which is used synonymously with the word "endotoxin." As used herein, TNFα means tumor necrosis factor alpha.

In one embodiment, the BPI variant has the structure $BPI_{(S351-}$

$_{>X)}$, serine residue 351 being substituted for X, an amino acid residue other than serine. In the preferred embodiment, X is alanine.

In this application, the portion of BPI in BPI variants and chimeras is designated by the letter B, followed by amino acid sequence numbers which correspond to those shown in Figure 5 for human BPI. Figure 5 designates the mature N-terminal amino acid as residue 1. The portion of LBP in LBP variants and chimeras is designated by the letter L, followed by amino acid sequence numbers which correspond to those shown in Figure 5 for human LBP. Figure 5 designates the mature N-terminal amino acid as residue 1.

For example, $L_{1-197}B_{200-456}$ (NCY118) contains amino acid residues 1-197 of LBP fused at its C-terminus to the N-terminus of BPI amino acid residues 200-456. $L_{1-197}B_{200-456}$ is shown in Figure 6. $L_{1-197}B_{200-456}$ has the N-terminal domain of LBP (having an endotoxin-binding domain) fused to the C-terminal domain of BPI (having a putative LPS-clearing domain).

In this application, single amino acid substitutions are noted in parentheses. The original amino acid residue (using the standard one letter code for amino acids), is followed by an arrow and the substitute amino acid residue. For example, in one BPI variant, original serine residue 351 is substituted with alanine (which removes a glycosylation signal) and is designated $BPI_{(S351->A)}$. As another example, the LBP-BPI chimera NCY103 is designated $L_{1-197(I43->V)}B_{200-456(N206->D)}$, which means that the original isoleucine residue 43 of the LBP portion is substituted with a valine residue, and the original asparagine residue 206 of BPI is substituted with aspartate. Suitable amino acid substitutions include but are not limited to substitutions of a particular amino acid

residue in one protein with the residue which resides at the corresponding position in a different protein. For example, $BPI_{(Xn->Y)}$ is a general designation for such a substitution. It means that amino acid residue X at position n in BPI is substituted with residue Y which is found at position n in LBP (or rabbit or bovine LBP). "X" and "Y" denote amino acid positions in a primary amino acid sequence. "Y" as used in this context is not to be confused with the symbol "Y" denoting the amino acid residue tyrosine. $LBP_{(Xn->Y)}$ is another example of such a substitution, wherein amino acid residue X at position n in LBP is substituted with residue Y which is found at position n in BPI (or rabbit or bovine BPI).

Amino acid residue insertions are also indicated in parentheses. First, the amino acid residue after which the insertion occurs and its number are given. After an arrow the amino acid residue before the insertion and the inserted amino acid are given. For example, in $B_{(DS200->DP)}$, a proline residue is substituted for the serine residue at position 200.

The subject invention also provides the BPI variant encoded by the recombinant nucleic acid molecule of the subject invention.

The subject invention further provides a vector comprising the recombinant cDNA molecule of the subject invention. Vectors not comprising the recombinant cDNA molecule of the subject invention are readily available to those skilled in the art, and can readily be used to form the vector of the subject invention.

Numerous vectors for expressing the inventive proteins may

be employed. Such vectors, including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses, are well known in the art. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow for the selection of transfected host cells. The markers may provide, for example, prototrophy to an auxotrophic host, biocide resistance or resistance to heavy metals such as copper. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. Additional elements may also be needed for optimal synthesis of mRNA. These additional elements may include splice signals, as well as enhancers and termination signals. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general.

The subject invention further provides a host vector system for the production of a BPI variant, which comprises the vector of the subject invention in a suitable host. Methods of producing host vector systems are well known to those skilled in the art.

Suitable host cells include, but are not limited to, bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to, HeLa cells, COS cells (including COS-7 cells), CV1 cells, NIH-3T3 cells, CHO cells, and Ltk cells. Certain animal cells, i.e., mammalian cells, may be transfected by methods well known in the art such as calcium phosphate precipitation, electroporation and microinjection.

In one embodiment, the suitable host is a bacterial cell. Bacterial cells include, for example, gram negative cells (e.g. E. coli cells). In another embodiment, the suitable host is an eucaryotic cell. The eucaryotic cell may be a mammalian cell. Mammalian cells include, for example, Chinese Hamster Ovary cells (CHO). The eucaryotic cell may also be a yeast cell. Yeast cells include, for example, Pichia cells.

The subject invention further provides a method for producing a BPI variant, which comprises growing the host vector system of the subject invention under conditions permitting the production of the BPI variant and recovering the BPI variant produced thereby.

Conditions permitting the production of the proteins in host vector systems are well known to those skilled in the art.

Protein recovery is accomplished by methods well known to those skilled in the art. Such methods include, but are not limited to, gel electrophoresis, ion exchange chromatography, affinity chromatography or combinations thereof.

The subject invention provides a recombinant nucleic acid molecule which encodes an LBP variant. In one embodiment, the recombinant nucleic acid molecule is a DNA molecule. In the preferred embodiment, the DNA molecule is a cDNA molecule.

As used herein, LBP or lipopolysaccharide binding protein means a protein having the amino acid sequence shown for human LBP in Figure 5. The amino acid sequence shown for human LBP in Figure 5 is distinct from the amino acid sequence reported by Schumann et al. (Science 249:1429-1431 (1990)). Therefore, the amino acid sequence shown for human LBP in Figure 5 should not be confused with the sequence reported by Schumann et al. Figure 1 shows differences between LBP sequence as used herein and LBP sequence as published by Schumann, et al. The LBP nucleotide and amino acid sequences are shown in Figure 4.

As used herein, an LBP variant means a protein comprising a portion of LBP, which protein is capable of (a) binding to LPS, (b) competing with BPI or LBP for binding to LPS, and (c) inhibiting the production of TNFα by human monocytes. An LBP variant may comprise, by way of example, a fragment of LBP, a point mutant of LBP, a deletion mutant of LBP, or a point and deletion mutant of LBP.

The subject invention provides a recombinant nucleic acid molecule which encodes an LBP-BPI chimera. In one embodiment, the recombinant nucleic acid molecule is a DNA molecule. In the preferred embodiment, the DNA molecule is

a cDNA molecule.

As used herein, a chimera means a protein comprising all or a portion of a first protein fused to all or a portion of a second protein, which resulting fusion protein may in turn be fused to all or a portion of a third protein. Chimeras include but are not limited to (a) a protein comprising a portion of LBP fused to a portion of BPI, (b) a protein comprising an LBP portion fused to a BPI portion which in turn is fused to a portion of an immunoglobulin, and (c) a protein comprising an LBP portion fused to a BPI portion, which in turn is fused to an LBP portion. Each protein portion of the chimera may comprise a fragment of the protein, a point mutant of the protein, a deletion mutant of the protein, or both a point and deletion mutant of the protein.

As used herein, an LBP-BPI chimera means a protein which (i) comprises an LBP portion fused to a BPI portion, and (ii) is capable of (a) binding to LPS, (b) competing with BPI or LBP for binding to LPS, and (c) inhibiting the production of TNFα by human monocytes.

Chimeras of LBP and BPI may share properties of both BPI and LBP. For example, fusing the N-terminal domain of LBP to the C-terminal domain of BPI results in an LBP-BPI chimera (e.g., NCY103 or NCY118). The resulting LBP-BPI chimera differs from LBP in that the chimera neutralizes endotoxin in whole blood and differs from BPI in that the chimera has a longer half-life in vivo. Such chimeras can be used to clear endotoxin from the blood of a patient with endotoxemia. A BPI-LBP chimera is a protein wherein all or a part of the N-terminal domain of BPI is fused to all or a part of the C-terminal domain of LBP (e.g., NCY104). This chimera competes effectively with BPI binding to endotoxin

but activates monocytes in the presence of endotoxin as does LBP.

For example, one or more of the nonconserved positively-charged residues in BPI (i.e., those residues not found at the corresponding positions in LBP) may be substituted with the corresponding residue or residues in LBP (as in, e.g., NCY139). Such substitutions would render BPI less cationic. As another example, one or more of the nonconserved amino acid residues in LBP (at a position which corresponds to a positively-charged residue in BPI) may be substituted with the corresponding positively-charged residue in BPI (as in, e.g., NCY141), and thus result in an LBP variant having an increased positive charge which enhances binding to the negatively charged phosphate groups in LPS, or facilitates interaction with the negatively charged surfaces of Gram-negative bacteria. Examples of positively-charged residues are lysine, arginine, and histidine.

Other BPI and LBP variants and chimeras have one or more cysteine residues deleted or substituted with serine or another amino acid. Such variants and chimeras help prevent the aggregation of BPI or LBP variants or chimeras during their production or use. For example, cysteine residue 132 in BPI (which is not conserved in LBP) is substituted with alanine (the corresponding residue in LBP) or serine.

Other BPI and LBP variants and chimera have one or more nonconserved glycosylation sites deleted (as in, e.g., NCY105) by amino acid substitution or deletion. Alternatively, a glycosylation site is added to other BPI and LBP variants and chimera by amino acid insertion or substitution.

Other BPI and LBP variants and chimera have one or more

secondary structure-altering amino acid residues deleted or added. For example, one or more of the nonconserved proline residues in BPI may be substituted with the corresponding non-proline residue in LBP. Alternatively, one or more of the nonconserved amino acid residues in LBP (at a position which corresponds to a proline in BPI) may be substituted with proline, which changes the secondary structure of LBP to become more like that of BPI.

In one embodiment, the LBP-BPI chimera has the structure $LBP_{1-197}BPI_{200-456}$. In still another embodiment, the LBP-BPI chimera has the structure $LBP_{1-197(I43->V)}BPI_{200-456(N206->D)}$.

In the preferred embodiment, the LBP-BPI chimera comprises all or a portion of the amino acid sequence of BPI from residue 199 to residue 359. The amino acid sequence of BPI from residue 199 to residue 359 contains a region required for neutralizing LPS, i.e., preventing LPS from stimulating an inflammatory response.

The subject invention also provides the recombinant nucleic acid molecule encoding the LBP-BPI chimera, vector and host vector system.

The subject invention provides a BPI-IgG chimera and a recombinant nucleic acid molecule which encodes a BPI-IgG chimera. In one embodiment, the recombinant nucleic acid molecule is a DNA molecule. In the preferred embodiment, the DNA molecule is a cDNA molecule.

As used herein, a BPI-IgG chimera means a protein which (i) comprises a BPI portion (at least 10 amino acid residues in length) fused at its C-terminus to the N-terminus of a portion of an IgG molecule; and (ii) is capable of (a)

binding to LPS, (b) competing with BPI or LBP for binding to LPS, and (c) inhibiting the production of TNFα by human monocytes. In the preferred embodiment, the portion of the IgG molecule is an IgG heavy chain Fc domain. The IgG heavy chain Fc domain may be the IgG heavy chain Fc domain whose sequence is shown in Figure 7. An example of a BPI-IgG chimera is $B_{1-199}Fc$.

The subject invention provides an LBP-IgG chimera and a recombinant nucleic acid molecule which encodes an LBP-IgG chimera. In one embodiment, the recombinant nucleic acid molecule is a DNA molecule. In the preferred embodiment, the DNA molecule is a cDNA molecule.

As used herein, an LBP-IgG chimera means a protein which (i) comprises an LBP portion (at least 10 amino acid residues in length) fused at its C-terminus to the N-terminus of a portion of an IgG molecule; and (ii) is capable of (a) binding to LPS, (b) competing with BPI or LBP for binding to LPS, and (c) inhibiting the production of TNFα by human monocytes.

The subject invention also provides the LBP-IgG chimera encoded by the recombinant nucleic acid molecule of the subject invention.

The subject invention further provides a vector comprising the recombinant cDNA molecule of the subject invention.

The subject invention provides an LBP-BPI-IgG chimera and a recombinant nucleic acid molecule which encodes an LBP-BPI-IgG chimera. In one embodiment, the recombinant nucleic acid molecule is a DNA molecule. In the preferred embodiment, the DNA molecule is a cDNA molecule.

As used herein, an LBP-BPI-IgG chimera means a protein which (i) comprises an LBP-BPI chimera fused at its C-terminus to the N-terminus of a portion of an IgG molecule; and (ii) is capable of (a) binding to LPS, (b) competing with BPI or LBP for binding to LPS, and (c) inhibiting the production of TNFα by human monocytes.

The BPI variant, LBP variant, LBP-BPI chimera, BPI-IgG chimera, LBP-IgG chimera, and LBP-BPI-IgG chimera of the subject invention may be modified with polyethylene glycol to increase the circulating half-life and/or bioavailability of the molecules.

The subject invention provides a pharmaceutical composition, which comprises a therapeutically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera; and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M succinate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Further, pharmaceutically acceptable carriers may include detergents, phospholipids, fatty acids, or other lipid carriers. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. A lipid carrier is any lipid-soluble

substance which inhibits protein precipitation and in which the proteins of the subject invention are soluble. Lipid carriers may be in the form of sterile solutions or gels. Lipid carriers may be detergents or detergent-containing biological surfactants. Examples of nonionic detergents include polysorbate 80 (also known as TWEEN 80 or polyoxyethylenesorbitan monooleate). Examples of ionic detergents include, but are not limited to, alykltrimethyl-ammonium bromide. Additionally, the lipid carrier may be a liposome. A liposome is any phospholipid membrane-bound vesicle capable of containing a desired substance, such as BPI or BPI variant, in its hydrophilic interior. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

The subject invention further provides a method of treating a subject suffering from an endotoxin-related disorder, which comprises administering to the subject a dose of the pharmaceutical composition of the subject invention effective to bind to LPS and thereby inhibit LPS biological activity.

As used herein, an endotoxin-related disorder includes, but is not limited to endotoxin-related shock, endotoxin-related disseminated intravascular coagulation, endotoxin-related anemia, endotoxin-related thrombocytopenia, endotoxin-related adult respiratory distress syndrome, endotoxin-related renal failure, endotoxin-related liver disease or hepatitis, SIRS (systemic immune response syndrome) resulting from Gram-negative infection, Gram-negative neonatal sepsis, Gram-negative meningitis, Gram-negative pneumonia, neutropenia and/or leucopenia resulting from

Gram-negative infection, hemodynamic shock and endotoxin-related pyresis. Endotoxin-related pyresis is associated with certain surgical procedures, such as trans-urethral resection of the prostate and gingival surgery. The presence of endotoxin may result from infection at any site with a Gram-negative organism, or conditions which may cause ischemia of the gastrointestinal tract, such as hemorrhage, or surgical procedures requiring extracorporeal circulation.

As used herein, the administeration may be performed by methods known to those skilled in the art. In one embodiment, the administeration comprises delivery to the lungs via an aerosol delivery system or via direct instillation. The aerosol may be nebulized. Other administeration modes include but are not limited to intravenous, intramuscular, and subcutaneous administration as well as direct delivery into an infected body cavity.

As used herein, the dose of the pharmaceutical composition of the subject invention effective to bind to LPS and thereby inhibit LPS-mediated stimulation of neutrophils and mononuclear cells is an amount sufficient to deliver to the subject an inventive protein at a concentration of between about 0.1mg/kg of body weight and about 100mg/kg of body weight. In one embodiment, the dose is an amount sufficient to deliver to the subject an inventive protein at a concentration of between about 1mg/kg of body weight and about 10mg/kg of body weight. The therapeutically effective amounts of inventive proteins in the pharmaceutical composition may be determined according to known methods based on the effective dosages discussed above.

As used herein, inhibit means to inhibit at a level which is statistically significant and dose dependent. The terms "statistically significant" and "dose dependent" are well

known to those skilled in the art.

The subject invention further provides a method of preventing an endotoxin-related disorder in a subject, which comprises administering to the subject a prophylactically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera, so as to thereby prevent the endotoxin-related disorder in the subject.

As used herein, a prophylactically effective amount is an amount between about 0.1mg/kg of body weight and about 100mg/kg of body weight. In the preferred embodiment, a prophylactically effective amount is an amount between about 1mg/kg of body weight and about 10mg/kg of body weight.

The term "inventive proteins" is used throughout the subject application. As used herein, the term "inventive proteins" means a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-LBP chimera, a BPI-IgG chimera, an LBP-IgG chimera, an LBP-BPI-IgG chimera, a recombinant protein comprising a portion of LBP or BPI, or any combination thereof.

The subject invention provides recombinant nucleic acid molecules which encode L_{1-199} (NCY109), $L_{1-357}B_{360-456}$ (NCY117), LBP (NCY102), $L_{1-199}Fc$ (NCY111), $L_{200-458}$ (NCY113), $LBP_{(A132->C)}$ (NCY126), $LBP_{(C61->F)}$ (NCY127), $LBP_{(C61->S)}$ (NCY128), $LBP_{(C135->S)}$ (NCY129), $LBP_{(A175->S)}$ (NCY130), $LBP_{C61->F)(C135->S)(A175->S)}$ (NCY131), or $LBP_{(C61->S)(C135->S)(A175->S)}$ (NCY132). In one embodiment, the recombinant nucleic acid molecules are DNA molecules. In the preferred embodiment, the DNA molecules are cDNA molecules. The subject invention also provides the proteins encoded by these recombinant nucleic acid molecules. The subject invention further provides vectors comprising these

recombinant cDNA molecules. The subject invention further provides host vector systems for the production of these proteins, which comprise these vectors in suitable hosts. In one embodiment, the suitable hosts are bacterial cells. In another embodiment, the suitable hosts are eucaryotic cells. The eucaryotic cells may be mammalian cells. The eucaryotic cells may also be yeast cells. The subject invention further provides methods for producing these proteins, which comprise growing these host vector systems under conditions permitting the production of these proteins and recovering the proteins produced thereby.

The proteins L_{1-199} (NCY109), $L_{1-357}B_{360-456}$ (NCY117), LBP (NCY102), L_{1-199}Fc (NCY111), $L_{200-458}$ (NCY113), $LBP_{(A132->C)}$ (NCY126), $LBP_{(C61->F)}$ (NCY127), $LBP_{(C61->S)}$ (NCY128), $LBP_{(C135->S)}$ (NCY129), $LBP_{(A175->S)}$ (NCY130), $LBP_{C61->F)(C135->S)(A175->S)}$ (NCY131), or $LBP_{(C61->S)(C135->S)(A175->S)}$ (NCY132) are useful for inhibiting the LPS-mediated cellular response both in vitro and in vivo.

Finally, the subject invention provides an article of manufacture comprising packaging material and a pharmaceutical composition contained within said packaging material wherein (a) the packaging material comprises a label which indicates that the pharmaceutical composition can be used for treating a subject suffering from an endotoxin-related disorder and for preventing endotoxin-related inflammation in a subject, and (b) said pharmaceutical composition comprises a therapeutically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera, and a pharmaceutically acceptable carrier.

These vectors may be introduced into a suitable host cell to

form a host vector system for producing the inventive proteins. Methods of making host vector systems are well known to those skilled in the art.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the subject inventions which follow thereafter.

Experimental Details

Materials and Methods

A series of BPI and LBP variants and chimeras are described in Tables 2 and 3. Table 2 describes some general classes of BPI and LBP variants and chimeras which are given by way of example. Specific examples of BPI and LBP variants and chimeras are described in Table 3 and are additionally designated by a product name (e.g., NCY103).

Table 2

Examples of BPI and LBP Variants and Chimeras

BPI variant (N-terminal frag.)	B_{1-n}
LBP variant (N-terminal frag.)	L_{1-n}
BPI variant (C-terminal frag.)	B_{n-456}
LBP variant (C-terminal frag.)	L_{n-456}
BPI variant (internal frag.)	B_{n-x}
LBP variant (internal frag.)	L_{n-x}
LBP-BPI chimera	$L_{n-x}B_{(x+1)-y}$
BPI-LBP chimera	$B_{n-x}L_{(x+1)-y}$
LBP-BPI chimera	$L_{n-x}B_{(x+1)-456}$
BPI-LBP chimera	$B_{n-x}L_{(x+1)-456}$
LBP-BPI chimera	$L_{1-n}B_{(n+1)-x}$
BPI-LBP chimera	$B_{1-n}L_{(n+1)-x}$
LBP-BPI chimera	$L_{1-n}B_{(n+1)-456}$
BPI-LBP chimera	$B_{1-n}L_{(n+1)-456}$
LBP-BPI-LBP chimera	$L_{1-n}B_{(n+1)-x}L_{(x+1)-456}$
BPI-LBP-BPI chimera	$B_{1-n}L_{(n+1)-x}B_{(x+1)-456}$

All of the above constructs could also be engineered as IgG chimeras. In such constructs, the Fc, or constant domain, or a human immunoglobulin heavy chain, can be linked to the BPI variant protein.

n represents an amino acid residue position in the mature sequence of BPI or LBP, x represents an amino acid residue in a position which is C-terminal to n in the sequence of BPI or LBP, and y represents an amino acid residue in a position which is C-terminal to x in the sequence of BPI or LBP. The symbols n, x and y denote the amino acid residue positions as they occur in the mature sequence of the native protein, and not necessarily the positions as they occur in the variant protein.

Table 3

Examples of BPI and LBP Variants and Chimeras

Sequence	Product Name	Description
BPI	NCY101	Native sequence
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	NCY103	LBP-BPI chimera
$B_{1-200}L_{199-456}$	NCY104	BPI-LBP chimera
$BPI_{(S351->A)}$	NCY105	Glycosylation site deleted
$BPI_{(DS200->DP)}$	NCY106	Acid cleavage site inserted
$L_{1-197}B_{200-456(S351->A)}$	NCY107	LBP-BPI chimera with glycoslation site deleted
B_{1-199}	NCY108	N-terminal domain of BPI
$B_{1-199}Fc$	NCY110	N-terminal BPI-IgG chimera
$B_{200-456}$	NCY112	C-terminal fragment of BPI

$L_{1-59}B_{60-456}$	NCY114	LBP-BPI chimera
$L_{1-134}B_{136-456}$	NCY115	LBP-BPI chimera
$L_{1-274}B_{277-456}$	NCY116	LBP-BPI chimera
$L_{1-359}B_{360-456}$	NCY117	LBP-BPI chimera
$L_{1-197}B_{200-456}$	NCY118	LBP-BPI chimera
$BPI_{(F61->C)}$	NCY119	Cysteine insertion
$BPI_{(C132->A)}$	NCY120	Cysteine deletion
$BPI_{(C132->S)}$	NCY121	Cysteine deletion
$BPI_{(C135->S)}$	NCY122	Cysteine deletion
$BPI_{(C-175->S)}$	NCY123	Cysteine deletion
$BPI_{(C132->A)(C135->S)(C175->S)}$	NCY124	Multiple cysteine deletion
$BPI_{(C-132->S)(C135->S(C175->S)}$	NCY125	Multiple cysteine deletion
$L_{(1-134)}B_{(136-361)}L_{(360-456)}$	NCY133	LBP-BPI chimera
$L_{(1-134)}B_{(136-275)}L_{(274-456)}$	NCY134	LBP-BPI chimera
$L_{(1-197)}B_{(200-275)}L_{(274-456)}$	NCY135	LBP-BPI chimera
$L_{(1-197)}B_{(200-361)}L_{(360-456)}$	NCY136	LBP-BPI chimera
$B_{(K27->S)(K30->L)(K33->T)}$ $_{(K42->R)(K44->P)(K48->R)(A59->H)}$	NCY137	Cationic Substit. (7)
$B_{(K77->S)(K86->R)(K90->R)}$ $_{(K96->S)(K118->L)(K127->R)}$	NCY138	Cationic Substit. (6)
$B_{(K148->G)(K150->D)(K160->N)}$ $_{(K161->Q)(R167->Q)(K169->V)}$ $_{(K177->M)(K185->D)(K197->E)}$	NCY139	Cationic Substit. (9)
$B_{(K77->S)(K86->R)(K90>R)}$ $_{(K96->S)(K118->L)(K127->R)(K148->G)(K150->D)(K160->N)}$ $_{(K161->Q)(R167->Q)(K169->V)(K177->M)}$ $_{(K185->D)(K197->E)}$	NCY140	Cationic Substit. (15)
$L_{(S77->K)(R86->K)(S96->K)}$ $_{(L118->K)(R126->K)}$	NCY141	Cationic Repl. (6)
$L_{(G147->K)(D148->K)(N158->K)}$ $_{(Q159->K)(Q165->R)(V167->K)(M175->K)(D183->K)}$ $_{(E196->K)}$	NCY142	Cationic Repl. (9)
$L_{(S77->K)(R86->K)(S96->K)}$ $_{(L118->K)(R126->K)(G147->K)(D148->K)(N158->K)(Q159->K)}$ $_{(Q165->R)(V167->K)(M175->K)(D183->K)}$ $_{(E196->K)}$	NCY143	Cationic Repl. (15)
$L_{(1-197)}B_{(200-456)}Fc$	NCY144	LBP-BPI-IgG chimera

Construction of Inventive proteins

The cDNA sequences of BPI and LBP are shown in Figures 3 and 4, respectively, with each nucleotide designated numerically. DNA encoding the inventive proteins was prepared by site-directed mutagenesis using standard techniques well known in the art [Zoller, M.J., et al., Methods Enzymol. 154:329 (1977)]. For example, the sequences "$ATAGAT_{723}$" and "$ATTGAC_{700}$" were chosen as a convenient site to insert a ClaI restriction site (ATCGAT) by which to recombine portions of BPI and LBP, respectively. Oligonucleotide primers were designed to overlap this region and to add the ClaI sequence, and were synthesized on an ABI 380B synthesizer (Applied Biosystems Inc., Foster City, CA). Additional primers were designed to bind to the 5' and 3'-ends of both molecules and to provide NheI (5') and XhoI (3') restriction sites for insertion into the vector. These primers were used to amplify portions of the cDNA molecules encoding amino acid residues 1-197 (A) and 200-456 (B) of LBP and BPI by cyclic DNA amplification. The resulting DNA fragments were digested with the appropriate restriction enzymes and then purified by gel electrophoresis.

Now that the useful LBP-BPI, BPI-IgG, LBP-IgG, and LBP-BPI-IgG chimeras have been disclosed, DNA molecules encoding these chimeras may be constructed using methods well known to those skilled in the art.

Mammalian Expression

In order to produce recombinant BPI, LBP, and the inventive proteins in mammalian cells, the cDNA sequences were inserted into a suitable plasmid vector. One suitable vector for such an application is pSE, which contains early and late promoters of SV40, followed by multiple insert cloning sites, followed by the termination sequences from

the hepatitis B surface antigen gene. Also contained within the plasmid are an origin of bacterial DNA replication, and the genes encoding ampicillin resistance and dihydrofolate reductase. Similar vectors have been used to express other foreign genes (McGrogan, et al. Biotechnology 6, 172-177). Another suitable vector, particularly for rapidly obtaining small quantities of inventive proteins was pCEP4 (Invitrogen Corp., San Diego, California). pCEP4 contains a CMV promoter, followed by multiple insert cloning sites, followed by SV40 termination sequences. Also contained within the plasmid are an origin of bacterial DNA replication, and the genes encoding resistance to ampicillin and hygromycin B. With pCEP4 and pSE, the same insert cloning sites as pSE for easy insert shuttling between the vectors were used. Once introduced into mammalian cell hosts, this specialized plasmid replicates as an episome, allowing semistable amplification of introduced DNA sequences. The high gene copy number is maintained through the selective pressure of culture in the presence of hygromycin B.

In both cases, vector DNA was prepared for acceptance of cDNA by digestion with Nhe I and Xho I, and was subsequently dephosphorylated by treatment with alkaline phosphatase. The prepared cDNA fragments encoding BPI, LBP, or other inventive proteins were ligated into pSE or pCEP4, and the resulting recombinant colonies were screened by agarose gel electrophoresis. Subsequently, the DNA sequences were confirmed by standard enzymatic sequencing methods (e.g., Sanger, 1974).

Expression plasmid DNA purified by either CsCl gradients with Plasmid or Midi Kits (Qiagen, Chatsworth, California) was used to transform Chinese hamster ovary strain DUKXB11 (pSE) and 293-EBNA cells (Invitrogen Corp., San Diego,

California) (pCEP4). Transfection was performed using lipofectin (Bethesda, Research Labs, Gaithersberg, MD) by standard methods. The resulting transformed cells were selected in GHT minus medium (DUKX11s) or in medium plus hygromycin B (293s). Cells were cultured in REM minus GHT plus 10% dialyzed fetal calf serum (DUKXB11s) or in REM and 10% calf serum (293s). For the DUKXB11s, clones were selected and were passed through sequential rounds of culture in increasing concentrations of methotrexate in order to amplify the DHFR gene and associated heterologous genes. Supernatants from transfected cells, either mixed populations or clones derived from the mixed population, were assayed for BPI, LBP, or inventive proteins by ELISA.

Yeast Expression

BPI and NCY118 were successfully expressed in the methylotrophic yeast Pichia pastoris. Pichia was chosen as a suitable expression system for BPI and BPI variants due to its lack of LPS (endotoxin to which BPI binds) and its ability to produce high levels of mammalian proteins.

P. pastoris strain GS115 (Invitrogen, San Diego, California) was transformed with plasmids encoding BPI and NCY118, and transformed colonies were selected for following the procedures outlined by Invitrogen (A Manual of Methods for Expression of Recombinant Proteins in Pichia pastoris, Version 1.5, Invitrogen, San Diego, California). For both BPI and NCY118, protein was secreted into the medium in a small-scale batch fermentation run. 116 ng/ml were secreted for the one BPI construct assayed, and 14, 11, and 10 ng/ml were secreted for the three NCY118 constructs assayed. Secretion was assayed by enzyme-linked immunosorbant analysis (ELISA). The majority of protein for both constructs was not secreted, as shown by Western blot analysis with a polyclonal anti-BPI antibody mix (INVN 1-2)

and alkaline phosphatase-conjugated goat anti-rabbit antibody. The Western blot is shown in Figure 19.

Purified BPI from Chinese Hamster ovary cells (CHOs) was used as a control (lane 12). In lane 1, was a sample from untransformed GS115 cells. The antibodies did not recognize any proteins from such cells subject to the detection limits of the assay. The next three lanes (2-4) were samples from colonies transformed with the construct for BPI and the last 6 lanes (5-10) were samples from colonies transformed with the construct for NCY118. The amount of intracellular BPI or NCY118 expressed in the batch fermentation run, based on the amount of standard BPI loaded, was roughly 100 ug/ml of medium for the BPI and NCY118 colonies.

Protein Purification

BPI (NCY101) was purified from conditioned media using the following four-step purification. BPI was captured on CM Sepharose (Pharmacia LKB Biotechnology). The column was washed in 50mM Tris pH 7.4, and protein was eluted with 50mM Tris buffer pH 7.4 + 1M NaCl. The eluate was diluted 10X with 50mM Tris pH 8.5, run over Fast Q Sepharose, and the flow through collected. BPI was re-captured on CM Sepharose, and again eluted as before. Buffer exchange into 10mM Succinate + 110mM NaCl pH 6 was performed using Sepharose CL6B (Pharmacia LKB Biotechnology). Finally, TWEEN 20 was added to the formulated material to a final concentration of 0.05%.

LBP (NCY102) was captured from cell culture medium on Fast S Sepharose (Pharmacia). The column was washed with 50mM Tris pH 7.4, and protein was eluted using 50mM Tris pH 7.4 + 1M NaCl. The eluate was diluted 10X in 50mM Tris pH 8.5, and run over HiLoad Q Sepharose (Pharmacia). Protein was eluted with a 0-1M NaCl gradient in 50mM Tris pH 8.5.

Appropriate fractions were pooled according to migration on SDS PAGE electrophoresis. NCY102 concentration was diluted to 4.0 mg/ml, and the pH adjusted to 7.0 with 100mM HCl.

NCY103 was purified from cell culture medium using the same method described for NCY102.

NCY104 and NCY105 were purified using the same protocol as for BPI, except that the size exclusion step was omitted.

NCY114, NCY115 and NCY138 were captured on a Poros II HS cation exchange column (PerSeptive Biosystems, Cambridge, MA) at pH 7.4. The column was washed with 20mM HEPES buffer at pH 7.5, and eluted with 20mM HEPES pH 7.5 with 1M NaCl. The eluate was diluted 5X in 20mM HEPES pH 7.5 and applied to a Poros HQ anion exchange column (PerSeptive) with the flow through applied directly to a POROS II HS column. The POROS II HS column was eluted with 3.3mM acetate, 3.3mM MES and 3.3mM HEPES, pH 6.5 with a 0-1M NaCl gradient.

NCY117 and NCY144 were captured from conditioned medium at pH 7.4 on a Poros II HS column. The column was washed with 20mM HEPES buffer at pH 7.5, and eluted with 20mM HEPES pH 7.5 + 1M NaCl. The eluate was diluted 10X with 20mM HEPES pH 7.5, loaded on a second, smaller Poros II HS column, and eluted with 3.3mM acetate, 3.3mM MES and 3.3mM HEPES, pH 6 with a 0-1M NaCl gradient.

Because purification of BPI, LBP and IgG are well known and purification of exemplary chimeras is described above, it is contemplated that those skilled in the art can purify additional BPI-IgG, LBP-IgG, and LBP-BPI-IgG chimeras of the subject invention by using the purification methods described above and/or by modifying these methods in ways familiar to those skilled in the art.

In Vitro and In Vivo Tests of Representative Compounds

In vitro and in vivo tests were performed on representative compounds disclosed herein. In vitro tests included LPS binding competition assay, Limulus amebocyte lysate (LAL) inhibition test, TNF release inhibition test, FITC-labeled LPS binding inhibition, THP-1 cell TNF production and BPI activity against Neisseria. In vivo tests included mouse LPS half-lives, mouse endotoxin challenges and LPS-induced cytokine function and mortality in rats, and LPS activation in bronchial fluids.

S. minnesota Re mutant LPS and FITC-labeled E. coli 055:B5 LPS were obtained from List Biological Laboratories (Campbell, CA). E. coli 0111:B4 LPS was obtained from Whitaker Biologicals (Walkersville, MD). S. abortus equi LPS was obtained from Sigma Chemical Co. (St. Louis, MO). HBSS without calcium and magnesium and Roswell Park Memorial Institute (RPMI solution) 1640 was obtained from Gibco BRL (Grand Island, MD). Fluorescent-activated cell sorting (FACS) analysis was performed on a FACStar, Becton Dickinson Immunocytometry Systems (Mountain View, CA).

^{biotinylated}BPI Binding Competition Assay

Binding to LPS immobilized on microtiter plates was performed using a modified procedure described by Ulevitch et al. (15). Briefly, Immulon 3 microtiter plates (96-well, Dynatech Biotechnology Products, Chantilly, VA) were coated with 1 or 4 μg of S. minnesota R595 Re LPS (LIST Biological Labs, Inc., #304) in 50mM borate, pH 9.5-9.8 + 20-25 mM EDTA overnight at 37°C. Blank, non-LPS coated wells were included on each plate and binding to these walls was used to determine non-specific binding. Absorbance values from wells which were not pre-coated with LPS consistently gave optical density readings of less than 0.05. Plates were

then washed extensively under running distilled deionized water, then dried at 37°C. All the wells were blocked for 60 minutes at 37°C with 1-2% very low endotoxin BSA (Sigma, St. Louis, MO) prepared in pyrogen-free Tris-buffered saline (50mM Tris pH 7.4 +150mM NaCl). The wells were emptied, and biotinylated BPI was incubated in the presence or absence of unlabeled BPI or inventive protein (pyrogen-free TBS + 1mg/ml low endotoxin BSA, and 0.05% Tween-20) was incubated in the LPS coated and uncoated wells for 2-3 hours at 37°C in a total volume of 100 µl/well. After four washes in assay buffer, plates were developed with streptavidin conjugated to alkaline phosphatase (BioRad, Burlingame, California) followed by 100 µl of PNP substrate solution (Sigma) freshly prepared from two 5 mg tablets dissolved in 10ml substrate buffer. Substrate buffer is prepared with 24.5 mg $MgCl_2$, 48 ml diethanolamine, brought up to 400 ml, pH adjusted to 9.8 and volume brought up to 500 ml. Absorbances were read at 405 nm on a Vmax kinetic microplate reader (Molecular Devices Inc., Menlo Park, CA).

Chromogenic LAL Assay

BPI and inventive proteins (25 µl of 0-200 µg/ml) were pre-incubated for 1 hour at 37°C with 1EU/ml of E. coli 0111:B4 LPS (25 µl of 2 EU/ml solution) (Whitaker Biologicals, Walkersville, Maryland). Then the mixtures were tested for LAL activity using the chromogenic LAL assay kit (Whitaker Biologicals, Walkersville, MD).

FITC-LPS Binding Assay

Blood collected in acid citrate dextrose-containing Vacutainer® tubes (Becton Dickinson, Rutherford, NJ) was diluted 1:4 in Hank's balanced salt solution (HBSS) minus calcium and magnesium. Mononuclear cells were isolated using Ficol-Paque (Pharmacia Inc., Piscataway, NJ). Cells

were washed three times in HBSS, then brought up to an appropriate volume of RPMI 1640 with glutamine and antibiotics to give approximately 1 X 10^6 cells/ml. To one ml aliquots of cells, FITC-LPS was added to a final concentration of 500 ng/ml. Tubes were closed and incubated at 37°C on a rocking platform. At the end of the incubation, cells were washed twice with PBS with 0.05% Human Serum Albumin (HSA) and 0.02% sodium azide. The monocyte portion of the cell population was determined by side scatter versus forward scatter gating and confirmed by staining a separate aliquot of cells with phycoerythrin-conjugated anti-DR antibody (Becton Dickinson Immunocytometry Systems, Milpitas, CA). Results are reported as logarithmic scale mean fluorescence intensity.

LPS-Induced TNF Release In Whole Blood

Peripheral blood from normal human volunteers was collected in heparin-containing Vacutainer- tubes (Becton Dickinson, Rutherford, NJ). To one milliliter of whole blood, BPI, an inventive protein, or buffer control was added, followed by 1ng/ml E. coli 055:B5 refined standard endotoxin (RSE) (Whitaker Bioproducts). Samples were incubated in closed microtubes at 37°C for 4 hours on a rocking platform. At the end of the incubation, samples were centrifuged for 5 minutes at 500xg at 4°C, the plasma collected and frozen on dry ice until assayed for the presence of cytokines. TNF levels were determined by ELISA using human recombinant TNF (Genzyme, Cambridge, MA or Genentech Inc., South San Francisco, CA) as a standard.

In later studies it was determined that BPI activity in whole blood is inhibited by heparin, and the anticoagulant was changed to citrate. In these experiments, to 120 µl of citrated whole blood, 20 µl or BPI or an inventive protein

(at 0-1 mg/ml) or buffer control, 20 μl of 100ng/ml of E. coli O55:B5 LPS was added to stimulate cells in whole blood samples. These experiments were performed in polypropylene microtiter plates (Costar, Cambridge, MA), which were centrifuged 15 min at 500 x g at 4°C.

THP-1 Cell TNF Production Assay

THP-1 cells were obtained from the American Tissue Culture Collection (Rockville, MD) and were maintained in REM medium containing 10% fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 μg/ml streptomycin. Cells were passed at 2 x 10^5 cells/ml every 3 days. Responsiveness of THP-1 cells to LPS was induced by culturing the cells for 48 hours in REM medium containing 10% fetal calf serum, 2mM L-glutamine, 100 units penicillin, 100 μg/ml of streptomycin and 100 nM PMA at 37°C in a humidified atmosphere with 5% CO_2. Cells were cultured in 96-well flat-bottomed tissue culture plates at 1-2 x 10^5 cells per well in a final volume of 200 μl. After 48 hours, adherent cells were washed three times with 200 μl of medium without serum. To 180 μl of medium without serum but with 0.5% HSA, LPS (10 μl at 200 ng/ml) and/or BPI, LBP or other inventive proteins were added (10 μl at 0-2 mg/ml) and the cells were cultured for an additional 4 hours. After 4 hours, supernatants were transferred to a U-bottomed 96 well plate and the plate was centrifuged (500 x g, 12 min.) to pellet any cell debris. Supernatants were then stored in a second plate at -20°C until assayed for TNF by ELISA.

Mouse Serum Half-Life Assay

CD-1 mice weighing approximately 20 grams were injected with 0.1 ml of BPI, LBP, or inventive protein (at 1 mg/ml) at time zero. In heparinized (or later EDTA-containing) tubes, blood was collected from the retroorbital plexus from three

animals at each time point tested. A typical blood collection schedule was 5, 10, 15, 30, 45, 60, 90, 120, 240, and 360 minutes. The blood was centrifuged for about 10 min at 1000 x g and the supernatant plasma frozen on dry ice until tested. Levels of BPI, LBP, or inventive protein in the plasma samples were determined by ELISA using the appropriate protein as the standard.

Mouse Endotoxin Challenge Assay

Female CD-1 mice were injected in the lateral tail vein with a LD_{100} dose (25-35 mg/kg) of Salmonella abortus equi endotoxin, which was followed by an injection of BPI, inventive protein, or vehicle control into the opposite lateral tail vein at the indicated time. Protein injection concentrations varied and provided doses of 0.5, 1 and 5 mg/kg. Use of vehicle control illustrated the effectiveness of the endotoxin challenge in the test animal. Animals were observed for mortality at 24, 48 and 72 hours.

BPI Reduction of LPS-Induced Cytokine Function and Mortality in Rats

The potential effect of NCY101 (BPI) against LPS-related cytokine formation and mortality was investigated in rats with either (a) hemorrhagic shock (bled to lower pressure to 30-35 mmHg mean arterial pressure for 90 minutes, followed by reinfusion of shed blood and an equal volume of Ringer's solution over 30 minutes), or (b) endotoxin shock (caused by 100μg LPS and 500mg D-galactosamine/kg). Treatment comprised 5mg BPI/kg i.v. for the BPI group, or 1ml saline i.v. for the control group.

BPI Activity Against N. meningitidis and N. gonorrhoeae

BPI suppresses TNF release by human inflammatory cells in response to lipopolysaccharide (LPS) derived from a wide

range of Gram-negative bacterial species. In order to test the activity of BPI against Gram-negative lipooligosaccharide (LOS) from the pathogenic bacteria Neisseria meningitidis and N. gonorrhoeae, non-viable bacteria were pre-treated with recombinant BPI and incubated with human whole blood for 4 hours at 37°C. Without BPI, N. meningitidis at 10^5 bacteria/ml stimulated the release of 2.93 ± 0.53 ng/ml of TNF, while N. gonorrhoeae was a more potent stimulator of TNF release; 10^4 bacteria/ml induced 8.23 ± 0.32 ng/ml of TNF. In both cases, 10μg/ml BPI completely inhibited TNF release. This indicates that BPI is able to bind and detoxify LOS of these organisms, as well as bind LPS. Thus, BPI may be useful as a therapeutic agent against LOS-mediated tissue damage associated with these pathogenic Neisseria species.

To compare the relative LPS binding affinities of BPI, LBP and inventive proteins, these proteins were tested for their ability to compete with 10ng/ml $^{\text{biotinylated}}$BPI for binding to LPS-coated microtiter plates as described supra. In these experiments, BPI inhibited $^{\text{biotinylated}}$BPI binding to LPS in a concentration-dependent manner (Figure 8). Modest inhibition of $^{\text{biotinylated}}$BPI-binding was observed using NCY102 (LBP) and NCY103, suggesting that BPI has either a higher affinity for LPS bound to a surface or that NCY102 and NCY103 bind to a different site on LPS. NCY104, which contains the N-terminal domain of BPI, competed with $^{\text{biotinylated}}$BPI at similar concentrations as unlabeled BPI, suggesting a similar affinity and binding site.

Competition between either NCY118 or NCY103 with biotinylated BPI occurred at similar concentrations, giving overlapping curves (Figure 14, panel A) indicating that the two amino acid differences between these two molecules

[NCY118->NCY103: (I43->V) and (N206->D)] had no effect on affinity for immobilized LPS. NCY144 (an IgG chimera consisting of NCY118 linked to human IgG1 Fc constant region of the immunoglobulin molecule) does not have an altered ability to compete with biotinylated BPI (Figure 14, panel A). NCY114 and NCY115 showed LPS affinity very similar to that observed for BPI, suggesting that the region between amino acid residues 1-59 (or 1-134) probably plays a minimal role in LPS binding (Figure 14, panel B). Together with data showing the NCY104 competes effectively with BPI (Figure 8), these results indicate that amino acid residues 134-197 are important structural components of the high-affinity LPS-binding domain of BPI.

The importance of the region between amino acid residues 134 to 197 in LPS affinity was further demonstrated by the markedly reduced affinity of NCY139, a mutant in which all of the cationic amino acids of the BPI molecule are replaced with the corresponding amino acid residues found in LBP. These changes resulted in a molecule with binding affinity for LPS which was more similar to that of LBP than BPI (Figure 14, panel C, and Figure 8). Amino acid residues 359 to 456 of BPI are not involved in LPS binding as demonstrated by the relative inability of NCY117 to displace biotinylated BPI from LPS (Figure 14, panel C). The apparent binding affinity of NCY117 for LPS is similar to that of LBP and NCY139, which affinity is approximately two orders of magnitude lower than the apparent affinity of BPI for LPS.

Thus, the domain of BPI which participates in binding to immobilized LPS is localized in the N-terminal half of the BPI molecule, since NCY104 has the greatest ability to displace native BPI from LPS coated onto microtiter plates. This domain of BPI has been more specifically localized to

a region between amino acid residues 134-199.

To test the relative abilities of BPI, LBP and inventive proteins to neutralize LPS in vitro, these proteins were tested for their ability to inhibit LPS in the chromogenic LAL assay (Figure 9 and Table 4). LPS was neutralized by the various proteins tested in the order of NCY105 ≥ BPI > NCY103 > NCY104 > NCY102. Several studies (shown as no. of tests) were carried out with different lots of each protein and the IC_{50} values were determined. The IC_{50} values were averaged and given in Table 4.

Table 4

LPS Inhibition in the Chromogenic LAL Assay

Product	I.C.$_{50}$ (μg/ml)	No. of tests
NCY105	1.5	(n=1)
BPI	5.2 ± 3.3	(n=10)
NCY103	28.0 ± 20.0	(n=4)
NCY104	40.0	(n=1)
NCY102	65.0 ± 31.0	(n=4)

These results demonstrate that BPI neutralizes LPS activity in the LAL assay at lower concentrations than LBP. NCY104, which contains the N-terminal domain of BPI, is a relatively poor inhibitor of LPS in the LAL assay. NCY103 was a more potent inhibitor than NCY102 (LBP) or NCY104. These results indicate that the N-terminal (LPS-binding) domain of BPI

alone does not account for the neutralizing activity of BPI in the LAL assay and that the C-terminal domain of BPI plays a very important role in endotoxin neutralization in the LAL assay.

Additional results of LPS neutralizing activity in the chromogenic LAL assay are shown in Table 5. NCY103, NCY114 and NCY115 share the C-terminal half of the BPI molecule, again indicating that this domain plays an important role in LPS-neutralizing activity. Also, these data indicate that the 199-456 region is most important in LPS neutralization since adding BPI amino acid residues between 136-456 or 60-456 did not improve LPS neutralizing activity. Together with the LPS binding data, these results further indicate that the C-terminal half of BPI is important for LPS neutralization, while the N-terminal sequence is more critical for LPS binding.

Table 5

LPS Inhibition in the Chromogenic LAL Assay

Protein		IC50	n
NCY101	Cumulative	1.95 ± 0.51	108
	Lot# 149718	1.57 ± 1.01	54
	Lot# 149719	1.69 ± 0.35	7
	Lot# 149722	1.70 ± 0.28	2
	Lot# 149724	1.41 ± 0.45	45
	Lot# 155794	1.95 ± 0.92	2
NCY102	Cumulative	55.92 ± 30.53	8
	Lot# 151281	34.33 ± 7.45	6
	Lot# 151204	77.50 ± 24.45	2
NCY103	Cumulative	22.86 ± 16.28	54
	Lot# 151235	25.50 ± 0.71	2
	Lot# 151242	36.50 ± 2.12	2
	Lot# 151274	3.46 ± 2.18	38
	Lot# 159616	8.83 ± 4.91	4

NCY104	Cumulative	24.19 ± 6.42	9
	Lot# 151246	12.50 ± 0.26	3
	Lot# 152658	10.70	1
	Lot# 155737	40.18 ± 34.48	4
NCY108	Cumulative	5.52 ± 5.05	17
	Lot# 151285	1.12 ± 0.00	2
	Lot# 155709	9.73 ± 1.18	3
	Lot# 155779	2.13 ± 0.81	2
NCY114	Lot# 155754	3.64 ± 1.64	5
NCY115	Lot# 155756	5.02 ± 3.11	5
NCY116	Lot# 155791	14.00 ± 2.65	3
NCY117	Lot# 155733	>100	4
NCY118	Cumulative	12.75 ± 3.54	12
	Lot# 155758	10.25 ± 30.9	8
	Lot# 159619	15.25 ± 5.91	4
NCY138	Lot# 155785	1.97 ± 0.06	3
NCY139	Lot# 155762	29.60 ± 23.23	5
NCY140	Lot# 155788	7.87 ± 2.80	3
NCY135	Lot# 159649	>100	3
NCY144	Lot# 155760	12.15 ± 6.00	4
NCY109		9.2	1
NCY108		10.1 ± 0.92	5
NCY134	Lot# 159643	22.00 ± 15.25	4

NCY139, which contains the entire BPI sequence except for nine cationic residues between positions 148 and 197, showed very poor LPS-neutralizing activity, suggesting that these residues are important in LPS-neutralizing activity. Similarly, this compound was relatively ineffective at LPS binding. These cationic residues may permit correct structural conformation of the molecule, since both NCY103 and NCY139 contain the C-terminal domain of BPI, yet NCY103

has potent neutralizing activity while NCY139 does not.

To determine the relative abilities of BPI and NCY103 to inhibit LPS binding to human peripheral blood monocytes, isolated human peripheral blood mononuclear cells were incubated with 10% human serum containing 500ng/ml FITC-conjugated E. coli 055:B5 LPS in the presence or absence of BPI or NCY103. Binding of FITC-LPS to monocytes could be inhibited by increasing concentrations of both BPI and NCY103 (Figure 10). Thus NCY103 has BPI-like binding activity, despite the fact that NCY103 contains the N-terminal domain of LBP. These data, along with the results of the LPS neutralization studies shown in Figure 9, suggest that the C-terminal domains of BPI and LBP, and not the N-terminal domains, determine whether the proteins inhibit or mediate LPS activation of cells.

Further studies were undertaken to determine the effects of BPI, LBP, NCY103 and NCY104 on FITC-labeled LPS binding to peripheral blood monocytes in the presence and absence of serum. In a serum-free FITC-labeled LPS binding system where no LBP is available, FITC-labeled LPS does not bind to cells. In contrast recombinant LBP facilitated LPS binding to cells at concentrations as low as 100ng/ml. NCY104 also facilitated binding, although to a lesser extent. Neither BPI or NCY103 promoted significant binding of LPS to cells. These data indicate that the C-terminal domain of LBP is active in LPS binding to cells. The N-terminal domain of BPI may exert an inhibitory influence on LPS binding to cells mediated by the C-terminal domain of LBP because NCY104 was less active than LBP.

Normal human serum contains about 1-10μg/ml LBP. In the presence of 10% autologous serum, BPI and NCY103 potently inhibited FITC LPS binding to monocytes, with BPI showing

slightly greater potency. NCY104 had marginal activity, and LBP had no effect (Figure 15, panel A). These data indicate that the C-terminal half of the BPI molecule was neutralizing LPS in this test. NCY104, which does not contain the C-terminal domain of BPI, is approximately two orders of magnitude less potent at blocking LPS binding in the presence of serum. LBP, as expected, had no effect. This demonstrated that BPI can intervene in the sepsis cascade by preventing LPS from binding to monocytes and causing release of TNFα.

To further identify the regions of BPI which contribute to LPS-neutralizing activity, and the domains of LBP which are responsible for transducing the LPS signal to cells, the abilities of inventive proteins to replace LBP were compared under serum-free conditions. In these experiments, cells of the promonocytic cell line THP-1 were induced to respond to LPS by culturing for 48 hours with phorbol ester. After induction, cells were stimulated with 19ng/ml of LPS in the presence or absence of the recombinant protein. In this system, no TNF is released without a source of LBP. Data from these experiments (Figure 16) show that only LBP and NCY117 stimulated TNF release. Thus the domain of LBP responsible for facilitating LPS-induced TNF release is within amino acid residues 199-357. Interestingly, NCY104 did not mediate TNF release in a serum-free system. This may indicate that the N-terminal domain of BPI binds too tightly to LPS to allow transfer of LPS to CD14 on the macrophage surface. Figure 17 shows an additional comparison of TNF production. NCY135, containing LBP domain 274-456, shows great activity, narrowing the active domain to 274-357.

To test the effects of BPI, LBP, and inventive proteins on LPS activation of TNF production in whole blood, BPI,

NCY102, NCY103, or NCY104 was mixed with heparinized blood, and LPS was added to the resulting mixture. The blood was incubated for four hours at 37°C, and TNF in the plasma was measured as described, supra. Results are shown in Figure 11. NCY103 was the most potent at blocking TNF release, followed by BPI as the next most potent blocker. NCY104 and LBP had essentially no effect. Thus, in whole blood, NCY103 proved to be the most potent inhibitor of LPS-mediated cytokine stimulation.

When experiments were performed in citrated rather than heparinized whole blood, endotoxin-neutralizing activity of BPI and NCY103 were equivalent (Table 6). In experiments in which recombinant proteins were preincubated with endotoxin before addition to whole blood, the activities of these compounds fell roughly into two groups. BPI, NCY103, NCY114, NCY115, and NCY118 possess LPS-neutralizing activity, while NCY104, NCY109 and NCY117 were relatively inactive. Results with NCY116, NCY139 and NCY144 were equivocal. When compounds were added to the blood samples immediately prior to LPS, the IC50 values were higher, but the same group of proteins showed activity. These data further indicate the role of the BPI carboxy terminal, particularly amino acid residues 200-359, in LPS neutralization in a highly physiological environment such as whole blood. Because NCY109 is not a potent endotoxin-neutralizing protein (see Tables 9 and 11), it can be concluded that the C-terminal domain of BPI must significantly contribute to the endotoxin-neutralizing activity of NCY103 and NCY118. All compounds which contain this sequence (200-359) are active except NCY139, which was also inactive in other assays, possibly because the replaced cationic amino acids help determine the correct structure of the molecule.

Table 6

LPS Inhibition in Human Whole Blood

Protein	IC50 (ug/ml) preincubated	n		IC50 (ug/ml) not preinc.	n
NCY115	0.15 ± 0.12	3	BPI	2.60 ± 1.52	5
NCY118	2.90 ± 3.59	12	NCY115	3.7 ± 1.60	2
NCY114	0.28 ± 0.25	3	NCY103	7.13 ± 5.92	4
NCY103	0.16 ± 0.11	17	NCY114	15 ± 18.58	2
BPI	0.43 ± 0.49	13	NCY118	26.5 ± 0.71	2
NCY144	18.00 ± 27.73	3	NCY117	>100	1
NCY104	>100	3	NCY139	>100	2
NCY117	>100	3	NCY144	>100	2
NCY139	11.50 ± 3.54	2*	NCY104	ND	
NCY108	0.73 ± 0.48	6	NCY108	4.0	1
NCY109	>100	2	NCY109	>100	1
NCY140	0.21 ± 0.26	3			
NCY138	0.27 ± 0.25	2			
NCY108	0.73 ± 0.48	6			
NCY134	2.0	1			
NCY135	5.27 ± 5.83	3			
NCY116	38.10 ± 53.64	3			

* Two additional values for NCY139 were >100.

A potent anti-endotoxin therapeutic should not only neutralize endotoxin, but should also have the capacity to clear endotoxin from the circulation. The circulating levels of radioactively labeled ^{125}I-BPI were measured in the mouse in the presence and absence of endotoxin (Table 7). In the absence of endotoxin, the elimination (alpha) phase for ^{125}I-BPI was less than two minutes. In the presence of LPS, the alpha phase was extended to 6.2 minutes. ^{125}I-LPS alone has a single phase distribution (beta) with a half-life of about 101 minutes. When ^{125}I-LPS and unlabeled BPI were administered, a 6.2 minute elimination (alpha) phase was observed, indicating that elimination was remarkably facilitated by BPI.

Table 7

Serum Half-Life of BPI and LPS in the Mouse

Test Article	t1/2alpha	t1/2beta
^{125}I-BPI	1.6	103.0
^{125}I-BPI + LPS	6.3	72.0
^{125}I-LPS	---	101.0
^{125}I-LPS + BPI	6.2	114.0

In order to determine whether the very short circulating half-life of BPI could be extended by molecular engineering, the circulating half-lives of BPI, LBP, NCY104 and NCY103 were compared (Figure 12). Using labeled material, it was observed that the circulating half-life of BPI in the mouse is remarkably short. This may result from the highly cationic nature of BPI which gives it a predicted pI of 10.6. LBP, normally present in the circulation at concentrations of 10μg/ml, has a predicted pI of about 6.8. As expected, NCY103 (LBP-BPI chimera lacking cationic residues) has a markedly longer circulating half-life than NCY104 (BPI-LBP chimera having cationic residues). Figure 12 shows that NCY103 indeed has a longer half-life than BPI. NCY104, with the N-terminal domain of BPI, had an even shorter half-life than that of BPI. Thus, the N-terminal domain of BPI appears to play a major role in its short circulating half-life.

Further pharmacokinetic studies were performed in which inventive proteins were administered to CD-1 mice at a 5mg/kg bolus dose. Results of these experiments are shown in Figure 18. At 5mg/kg, the circulating half life of NCY104 was similar to that of BPI. NCY103 and NCY118 had

overlapping elimination curves and persisted in the circulation significantly longer than BPI or NCY104, but not as long as the serum protein LBP. Comparison of the elimination curves of NCY114, NCY115 and NCY139 revealed that the N-terminus of LBP plays a role in extending circulating half-life. NCY114 circulates slightly longer than BPI and contains the least LBP sequence of any of the recombinant proteins tested (amino acid residues 1-59). NCY115 was cleared somewhat more slowly, indicating that LBP amino acid residues 60-134 impart a longer circulating half-life. In contrast, the cationic residues of BPI between amino acid residues 134-199 shorten the half-life, since in NCY139, where the cationic residues in this region were replaced with the corresponding residues of LBP, the half-life was similar to that of NCY115. Including more LBP residues in the N-terminal domain further extends the half life. If amino acid residues 199-357 of LBP are added (NCY117) the half-life is longer, but not quite as long as that of LBP. Likewise NCY135 (with LBP domain 1-199 and 274-456 has a relatively long $T_{1/2}$. These results indicate that the more "LBP-like" the molecule is, the longer it circulates. In addition, combining an Ig fragment F_c with NCY103 gives the longest half life.

The efficacies of BPI, LBP, NCY103, NCY104 and NCY105 against lethal endotoxin challenge in mice were compared (Tables 8-10). The efficacies of NCY103, NCY118, NCY114, NCY115, NCY144, NCY116, NCY117, NCY139, NCY138 and NCY140 against lethal endotoxin challenge in mice were also compared (Table 11). When each protein was given within two minutes after lethal endotoxin challenge, BPI, NCY103 and NCY105 had similar potency, whereas LBP and NCY104 showed modest but incomplete protection and were not as effective as BPI. The partial protective effects of LBP and NCY104

may reflect species differences betweens humans and mice, since these agents do not block the inflammatory signal of LPS acting on human cells in vitro (Figure 11).

Table 8

Mouse Endotoxin Challenge
Comparison of BPI, NCY102 and NCY103

Drug	Dose of BPI or Variant	% Survival (n=10)
Control	0	0%
BPI	5mg/kg	60%
	1mg/kg	40%
NCY102	5mg/kg	30%
	1mg/kg	20%
NCY103	5mg/kg	60%
	1mg/kg	50%

Table 9

Mouse Endotoxin Challenge
Comparison of BPI, NCY103 and NCY105

Drug	Dose of BPI or Variant	% Survival (n=10)
Control	0	0%
BPI	5 mg/kg	80%
NCY103	5 mg/kg	100%
NCY105	5 mg/kg	90%

Table 10

Mouse Endotoxin Challenge
Comparison of BPI and NCY104

Drug	Dose of BPI or Variant	% Survival (n=10)
	0	40%
BPI	10mg/kg	100%
	2 mg/kg	100%
	0.4mg/kg	70%
NCY104	10mg/kg	60%
	2mg/kg	60%
	0.2mg/kg	50%

Table 11

Survival in CD-1 Mice Following
Lethal Endotoxin Challenge

Panel A

	Survivors/n	% Survival	p (vs. control)
BPI	40/50	80.00	< 0.001
NCY103	17/20	85.00	< 0.001
NCY118	16/20	80.00	< 0.001
NCY114	13/20	65.00	< 0.001
NCY115	13/20	65.00	< 0.001
NCY144	5/10	50.00	0.002
NCY117	2/10	20.00	0.149
NCY139	1/10	10.00	0.442
NCY116	0/10	0	--
NCY138	9/10	90.00	< 0.001
NCY140	6/10	60.00	< 0.05
Control	1/30	3.30	--

Panel B

	Dose mg/kg	Survivors (n=20)	% Survival	p (vs. control)*
BPI	5	13	65	< 0.001
	1	9	45	0.001
	0.5	6	30	0.02
NCY103	5	18	90	<0.001
	1	12	60	<0.001
	0.5	9	45	0.001
NCY108	5	3	15	NS
	1	0	0	NS
	0.5	1	5	NS

* Fisher's Exact Test

NCY103 was markedly more effective than BPI when given more than an hour before or after LPS (Figure 13). These results indicate that the longer circulating half-life of NCY103, or perhaps the increased ability of NCY103 to inhibit endotoxin in whole blood, has a dramatic effect on NCY103 efficacy in vivo.

Further experiments were performed to assess the LPS-neutralizing activities of inventive proteins in vivo. In these experiments, a lethal LPS challenge was administered at time zero, followed immediately by a 5mg/kg bolus injection of recombinant protein.

The potential effect of NCY101 (BPI) against LPS related cytokine formation and mortality was investigated in rats with either (a) hemorrhagic shock or (b) endotoxin shock. The important role of endotoxin in hemorrhage (with endogenous LPS translocation from the gut), trauma and sepsis is well known. BPI binds LPS and inhibits LPS-

mediated neutrophil and monocyte stimulation. Similarly, recombinant BPI binds LPS and inhibits TNF formation in vitro.

The results of the investigation of BPI efficacy in rats with either (a) hemorrhagic shock or (b) endotoxin shock show that (a) in rats with hemorrhagic shock, the mortality was decreased from 5/10 (50% control group) to 2/10 (20% BPI group) at 48 hours; (b) in rats with endotoxin shock, the 5-day mortality was significantly reduced ($p = 0.055$) by BPI treatment to 43%, as compared to 83% in the control group. Plasma LPS levels were at least partially neutralized at two hours (5.9 ± 4.1 vs 10.8 ± 4.1ng/ml). Cytokine formation was concomitantly reduced in the BPI group as measured by plasma TNF levels at two hours (3.9 ± 2.9 vs 10.3 ± 6.3ng/ml). Liver Transaminases (GOT and GPT, whose elevation indicates hepatic dysfunction) and bilirubin still increased at eight hours; however, the increase was less with BPI. These data demonstrate that BPI might be a useful therapeutic agent against endotoxin-related disorders in hemorrhagic and endotoxin shock.

Anesthetized male CD-1 mice were treated intra-nasally with 1 or 10μg of either BPI or NCY103 in 50μl. Control animals received 50μl of saline for injection. After 20 minutes, animals were re-anesthetized, and challenged with 10ng of E. coli O55:B5 LPS. One hour after endotoxin challenge, mice were re-anesthetized, and 0.7ml of saline containing 1% human serum albumin was added to the lungs via the trachea. The lungs were gently kneaded. A 0.5ml volume of BAL (bronchoalveolar lavage) fluid was aspirated, cells were pelleted by centrifugation, and the BAL sample was stored at -70°C. The TNF-alpha level in the BAL fluid was determined by ELISA (results shown in Figure 20).

Figure 20 shows that endotoxin-neutralizing proteins such as BPI and NCY103 can also neutralize endotoxin-mediated TNF release in the lung. These results indicate that these proteins are effective when delivered directly into the lung. This supports use in the treatment of pneumonias and other endotoxin-related disorders of the lung, such as ARDS.

CLAIMS

1. A recombinant nucleic acid molecule which encodes a BPI variant.

2. The recombinant nucleic acid molecule of claim 1, wherein the BPI variant has the structure $BPI_{(S351->X)}$, wherein X is alanine or an amino acid residue other than serine.

3. A recombinant nucleic acid molecule which encodes an LBP variant.

4. A recombinant nucleic acid molecule which encodes an LBP-BPI chimera.

5. The recombinant nucleic acid molecule of claim 4, wherein the LBP-BPI chimera has the structure $LBP_{1-197}BPI_{200-456}$.

6. The recombinant nucleic acid molecule of claim 4, wherein the LBP-BPI chimera has the structure $LBP_{1-197(I43->V)}BPI_{200-456(N206->D)}$.

7. A recombinant nucleic acid molecule which encodes a BPI-IgG chimera.

8. A recombinant nucleic acid molecule which encodes an LBP-IgG chimera.

9. A recombinant nucleic acid molecule which encodes an LBP-BPI-IgG chimera.

10. The recombinant nucleic acid molecule of claims 1 through 9, wherein the nucleic acid molecule is a DNA molecule.

11. The polypeptide encoded by the recombinant nucleic acid molecule of claims 1 through 9.

12. A vector comprising the recombinant nucleic acid molecule of claims 1 through 9.

13. A host vector system for the production of a BPI variant, which comprises the vector of claim 12 in a suitable host.

14. The host vector system of claim 13, wherein the suitable host is a bacterial or mammalian cell.

15. A method for producing a variant polypeptide, which comprises growing the host vector system of claim 13 under conditions permitting the production of the variant polypeptide and recovering the variant polypeptide produced thereby.

16. A pharmaceutical composition, which comprises a therapeutically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera, and a pharmaceutically acceptable carrier.

17. A method of treating a subject suffering from an endotoxin-related disorder, which comprises administering to the subject a dose of the pharmaceutical composition of claim 16 effective to bind to LPS and thereby inhibit LPS-mediated stimulation of neutrophils and mononuclear cells,

so as to thereby treat the subject.

18. A method of preventing an endotoxin-related disorder in a subject, which comprises administering to the subject a prophylactically effective amount of a BPI variant, an LBP variant, an LBP-BPI chimera, a BPI-IgG chimera, an LBP-IgG chimera, or an LBP-BPI-IgG chimera.

FIGURE 1A

LBP-a vs b

```
                                                              -30                  -20                 -10
Mouse   LBP                                                                 l p s t l l g l l f l s i q s i g s
Human   LBP a                                       . . . . . . m g a l a h a - l p s i l l a l l l l s i p e s l g
Human   LBP b                                       . . . . . . m g a l a h a - l p s i l l a l l l l s i p e a l g

                            10                  20                  30                  40                  50
Rabbit  LBP     I N P G I I T R I T D K G L E Y A A R E G L L A L Q R K L L E V T L P D S D G D F R I K H F G R A Q
Human   LBP a   A N P G L V A R I T D K G L Q Y A A Q E G L L A L Q S E L L R I T L P D F T G D L R I P H V G R G R
Human   LBP b   A N P G L V A R I T D K G L Q Y A A Q E G L L A L Q S E L L R I T L P D F T G D L R I P H V G R G R

                            60                  70                  80                  90                  100
Rabbit  LBP     Y K F Y S L K I P R F E L L R G T L R P L P G Q G L S L D I S D A Y I H V R G S W K V R K A F L R L
Human   LBP a   Y E F H S L N I H S C E L L H S A L R P V P G Q G L S L S I S D S S I R V Q G R W K V R K S F F K L
Human   LBP b   Y E F H S L N I H S C E L L H S A L R P V P G Q G L S L S I S D S S I R V Q G R W K V R K S F F K L

                            110                 120                 130                 140                 150
Rabbit  LBP     K N S F D L Y V K G L T I S V H L V L G S E - S S G R P T V T T S S C S S D I Q N V E L D I E G - D
Human   LBP a   Q G S F D V S V K G I S I S V N L L L G S E - S S G R P T G C Y L S C S S D I A D V E V D M S G - D
Human   LBP b   Q G S F D V S V K G I S I S V N L L L G S E - S S G R P T V T A S S C S S D I A D V E V D M S G - D

                            160                 170                 180                 190                 250/300
Rabbit  LBP     L E E L L N L L Q S Q I D A R L R E V L E S K I C R Q I E E A V T A H L Q P Y L Q T L P V T T Q I D
Human   LBP a   S G W L L N L F H N Q I E S K F Q K V L E S R I C E M I Q K S V S S D L Q P Y L Q T L P V T T E I D
Human   LBP b   F G W L L N L F H N Q I E S K F Q K V L E S R I C E M I Q K S V S S D L Q P Y L Q T L P V T T E I D

                            210                 220                 230                 240                 250
Rabbit  LBP     S F A G I D Y S L M E A P R A T A G M L D V M F K G E I F P L D H R S P V D F L A P A M N L P E A H
Human   LBP a   S F A D I D Y S L V E A P R A T A Q M L E V M F K G E I F H R N H R S P V T L L A A A - - - - - E E H
Human   LBP b   S F A D I D Y S L V E A P R A T A Q M L E V M F K G E I F H R N H R S P V T L L A A V M S L P E E H

                            260                 270                 280                 290                 300
Rabbit  LBP     S R M V Y F S I S D Y V F N T A S L A Y H K S G Y W N F S I T D A M V P A D L N I R R T T K S F R P
Human   LBP a   N K M V Y F A I S D Y V F N T A S L V Y H E E G Y L N F S I T D D M I P P D S N I R L T T K S F R P
Human   LBP b   N K M V Y F A I S D Y V F N T A S L V Y H E E G Y L N F S I T D D M I P P D S N I R L T T K S F R P

                            310                 320                 330                 340                 350
Rabbit  LBP     F V P L L A N L Y P N M N L E L Q G T V N S E Q L V N L S T E N L L E E P E M D I E A L V V L P S S
Human   LBP a   F V P R L A R L Y P N M N L E L Q G S V P S A P L L N F S P G N L S V D P Y M E I D A F V L L P S S
Human   LBP b   F V P R L A R L Y P N M N L E L Q G S V P S A P L L N F S P G N L S V D P Y M E I D A F V L L P S S

                            360                 370                 380                 390                 400
Rabbit  LBP     A R E P V F R L G V A T N V S A T L T L N T R K I T G F L K P G R L Q V E L K E S K V G G F N V E L
Human   LBP a   S K E P V F R L S V A T N V S A T L T F N T S K I T G F L K P G K V K V E L K E S K V G L F N A E L
Human   LBP b   S K E P V F R L S V A T N V S A T L T F N T S K I T G F L K P G K V K V E L K E S K V G L F N A E L

                            410                 420                 430                 440                 450
Rabbit  LBP     L E A L L N Y Y I L N N L Y P K V N E K L A H R F P L P L L R H I Q L Y D L L L Q T H E N F L L V G
Human   LBP a   L E A L L N Y Y I L N T L Y P K F N D K L A E G F P L P L L K R V Q L Y D L G L Q I H K D F L F L G
Human   LBP b   L E A L L N Y Y I L N T F Y P K F N D K L A E G F P L P L L K R V Q L Y D L G L Q I H K D F L F L G

                      458
Rabbit  LBP     A N I Q Y R R V
Human   LBP a   A N V Q Y M R V
Human   LBP b   A N V Q Y M R V
```

FIGURE 1B

INDIVIDUAL SEQUENCE DIFFERENCES
(LBP-A vs. LBP-B)

Nucleic Acid		Protein	
Alpha	*Beta*	*Alpha*	*Beta*
A_{42}	C_{42}		
C_{318}	T_{318}		
G_{488}	(np)	$_{154}GYCL_{157}$	$_{154}VTAS_{157}$
(np)	C_{499}		
T_{546}	C_{546}		
C_{548}	T_{548}	S_{174}	L_{174}
(np)	$_{824}TCATGAGCCTTC_{835}$	A_{266}	$_{266}VMSLP_{270}$
C_{1333}	T_{1333}	L_{436}	F_{436}

(np) = not present in the sequence

Figure 2

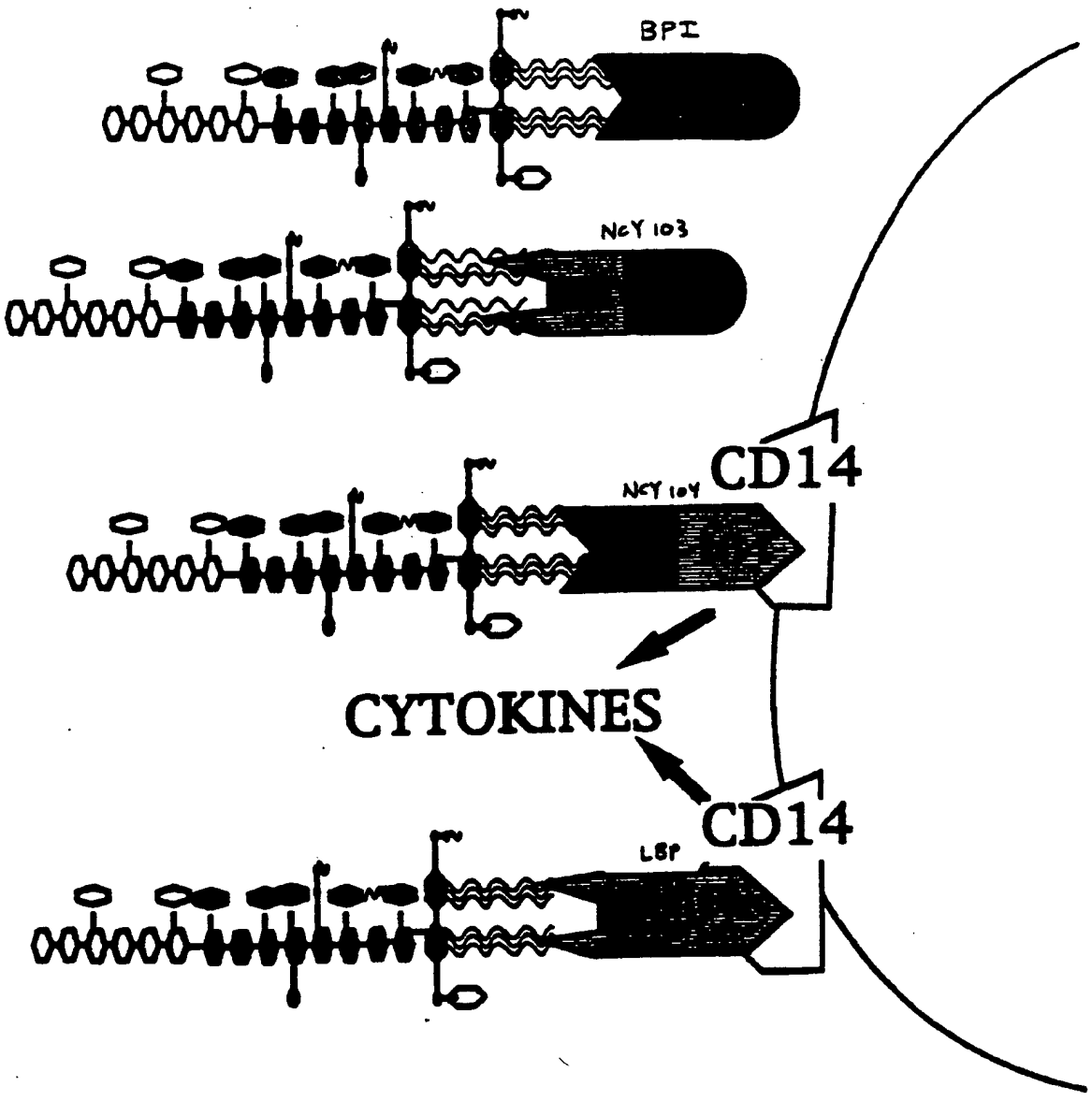

FIGURE 3A

BPI cDNA

```
   1  CAG GCC TTG AGG TTT TGG CAG CTC TGG AGG ATG AGA GAG AAC ATG GCC   48
   1                                          Met Arg Glu Asn Met Ala    6

  49  AGG GGC CCT TGC AAC GCG CCG AGA TGG GTG TCC CTG ATG GTG CTC GTC   96
   7  Arg Gly Pro Cys Asn Ala Pro Arg Trp Val Ser Leu Met Val Leu Val   22

  97  GCC ATA GGC ACC GCC GTG ACA GCG GCC GTC AAC CCT GGC GTC GTG GTC  144
  23  Ala Ile Gly Thr Ala Val Thr Ala Ala Val Asn Pro Gly Val Val Val   38

 145  AGG ATC TCC CAG AAG GGC CTG GAC TAC GCC AGC CAG CAG GGG ACG GCC  192
  39  Arg Ile Ser Gln Lys Gly Leu Asp Tyr Ala Ser Gln Gln Gly Thr Ala   54

 193  GCT CTG CAG AAG GAG CTG AAG AGG ATC AAG ATT CCT GAC TAC TCA GAC  240
  55  Ala Leu Gln Lys Glu Leu Lys Arg Ile Lys Ile Pro Asp Tyr Ser Asp   70

 241  AGC TTT AAG ATC AAG CAT CTT GGG AAG GGG CAT TAT AGC TTC TAC AGC  288
  71  Ser Phe Lys Ile Lys His Leu Gly Lys Gly His Tyr Ser Phe Tyr Ser   86

 289  ATG GAC ATC CGT GAA TTC CAG CTT CCC AGT TCC CAG ATA AGC ATG GTG  336
  87  Met Asp Ile Arg Glu Phe Gln Leu Pro Ser Ser Gln Ile Ser Met Val  102

 337  CCC AAT GTG GGC CTT AAG TTC TCC ATC AGC AAC GCC AAT ATC AAG ATC  384
 103  Pro Asn Val Gly Leu Lys Phe Ser Ile Ser Asn Ala Asn Ile Lys Ile  118

 385  AGC GGG AAA TGG AAG GCA CAA AAG AGA TTC TTA AAA ATG AGC GGC AAT  432
 119  Ser Gly Lys Trp Lys Ala Gln Lys Arg Phe Leu Lys Met Ser Gly Asn  134

 433  TTT GAC CTG AGC ATA GAA GGC ATG TCC ATT TCG GCT GAT CTG AAG CTG  480
 135  Phe Asp Leu Ser Ile Glu Gly Met Ser Ile Ser Ala Asp Leu Lys Leu  150

 481  GGC AGT AAC CCC ACG TCA GGC AAG CCC ACC ATC ACC TGC TCC AGC TGC  528
 151  Gly Ser Asn Pro Thr Ser Gly Lys Pro Thr Ile Thr Cys Ser Ser Cys  166

 529  AGC AGC CAC ATC AAC AGT GTC CAC GTG CAC ATC TCA AAG AGC AAA GTC  576
 167  Ser Ser His Ile Asn Ser Val His Val His Ile Ser Lys Ser Lys Val  182

 577  GGG TGG CTG ATC CAA CTC TTC CAC AAA AAA ATT GAG TCT GCG CTT CGA  624
 183  Gly Trp Leu Ile Gln Leu Phe His Lys Lys Ile Glu Ser Ala Leu Arg  198

 625  AAC AAG ATG AAC AGC CAG GTC TGC GAG AAA GTG ACC AAT TCT GTA TCC  672
 199  Asn Lys Met Asn Ser Gln Val Cys Glu Lys Val Thr Asn Ser Val Ser  214

 673  TCC AAG CTG CAA CCT TAT TTC CAG ACT CTG CCA GTA ATG ACC AAA ATA  720
 215  Ser Lys Leu Gln Pro Tyr Phe Gln Thr Leu Pro Val Met Thr Lys Ile  230

 721  GAT TCT GTG GCT GGA ATC AAC TAT GGT CTG GTG GCA CCT CCA GCA ACC  768
 231  Asp Ser Val Ala Gly Ile Asn Tyr Gly Leu Val Ala Pro Pro Ala Thr  246

 769  ACG GCT GAG ACC CTG GAT GTA CAG ATG AAG GGG GAG TTT TAC AGT GAG  816
 247  Thr Ala Glu Thr Leu Asp Val Gln Met Lys Gly Glu Phe Tyr Ser Glu  262

 817  AAC CAC CAC AAT CCA CCT CCC TTT GCT CCA CCA GTG ATG GAG TTT CCC  864
 263  Asn His His Asn Pro Pro Pro Phe Ala Pro Pro Val Met Glu Phe Pro  278

 865  GCT GCC CAT GAC CGC ATG GTA TAC CTG GGC CTC TCA GAC TAC TTC TTC  912
 279  Ala Ala His Asp Arg Met Val Tyr Leu Gly Leu Ser Asp Tyr Phe Phe  294

 913  AAC ACA GCC GGG CTT GTA TAC CAA GAG GCT GGG GTC TTG AAG ATG ACC  960
 295  Asn Thr Ala Gly Leu Val Tyr Gln Glu Ala Gly Val Leu Lys Met Thr  310

 961  CTT AGA GAT GAC ATG ATT CCA AAG GAG TCC AAA TTT CGA CTG ACA ACC 1008
 311  Leu Arg Asp Asp Met Ile Pro Lys Glu Ser Lys Ph  Arg Leu Thr Thr  326

1009  AAG TTC TTT GGA ACC TTC CTA CCT GAG GTG GCC AAG AAG TTT CCC AAC 1056
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FIGURE 3B

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 327  Lys Phe Phe Gly Thr Phe Leu Pro Glu Val Ala Lys Lys Phe Pro Asn      342

1057  ATG AAG ATA CAG ATC CAT GTC TCA GCC TCC ACC CCG CCA CAC CTG TCT     1104
 343  Met Lys Ile Gln Ile His Val Ser Ala Ser Thr Pro Pro His Leu Ser      358

1105  GTG CAG CCC ACC GGC CTT ACC TTC TAC CCT GCC GTG GAT GTC CAG GCC     1152
 359  Val Gln Pro Thr Gly Leu Thr Phe Tyr Pro Ala Val Asp Val Gln Ala      374

1153  CTT GCC GTC CTC CCC AAC TCC TCC CTG GCT TCC CTC TTC CTG ATT GGC     1200
 375  Leu Ala Val Leu Pro Asn Ser Ser Leu Ala Ser Leu Phe Leu Ile Gly      390

1201  ATG CAC ACA ACT GGT TCC ATG GAG GTC AGC GCC GAG TCC AAC AGG CTT     1248
 391  Met His Thr Thr Gly Ser Met Glu Val Ser Ala Glu Ser Asn Arg Leu      406

1249  GTT GGA GAG CTC AAG CTG GAT AGG CTG CTC CTG GAA CTG AAG CAC TCA     1296
 407  Val Gly Glu Leu Lys Leu Asp Arg Leu Leu Leu Glu Leu Lys His Ser      422

1297  AAT ATT GGC CCC TTC CCG GTT GAA TTG CTG CAG GAT ATC ATG AAC TAC     1344
 423  Asn Ile Gly Pro Phe Pro Val Glu Leu Leu Gln Asp Ile Met Asn Tyr      438

1345  ATT GTA CCC ATT CTT GTG CTG CCC AGG GTT AAC GAG AAA CTA CAG AAA     1392
 439  Ile Val Pro Ile Leu Val Leu Pro Arg Val Asn Glu Lys Leu Gln Lys      454

1393  GGC TTC CCT CTC CCG ACG CCG GCC AGA GTC CAG CTC TAC AAC GTA GTG     1440
 455  Gly Phe Pro Leu Pro Thr Pro Ala Arg Val Gln Leu Tyr Asn Val Val      470

1441  CTT CAG CCT CAC CAG AAC TTC CTG CTG TTC GGT GCA GAC GTT GTC TAT     1488
 471  Leu Gln Pro His Gln Asn Phe Leu Leu Phe Gly Ala Asp Val Val Tyr      486

1489  AAA TGA AGG CAC CAG GGG TGC CGG GGG CTG TCA GCC GCA CCT GTT CCT     1536
 487  Lys ***                                                            488

1537  GAT GGG CTG TGG GGC ACC GGC TGC CTT TCC CCA GGG AAT CCT CTC CAG     1584

1585  ATC TTA ACC AAG AGC CCC TTG CAA ACT TCT TCG ACT CAG ATT CAG AAA     1632

1633  TGA TCT AAA CAC GAG GAA ACA TTA TTC ATT GGA AAA GTG CAT GGT GTG     1680

1681  TAT TTT AGG GAT TAT GAG CTT CTT TCA AGG GCT AAG GCT GCA GAG ATA     1728

1729  TTT CCT CCA GGA ATC GTG TTT CAA TTG TAA CCA AGA AAT TTC CAT TTG     1776

1777  TGC TTC ATG AAA AAA AAC TTC TGG TTT TTT TCA TGT G                   1813
```

FIGURE 4A

Human LBP Expression clone

```
   1  GCT AGC CCA CTG CAC TGG GAA TCT AGG ATG GGG GCC TTG GCC AGA GCC   48
   1   NheI                               Met Gly Ala Leu Ala Arg Ala    7

  49  CTG CCG TCC ATA CTG CTG GCA TTG CTG CTT ACG TCC ACC CCA GAG GCT   96
   8  Leu Pro Ser Ile Leu Leu Ala Leu Leu Leu Thr Ser Thr Pro Glu Ala   23

  97  CTG GGT GCC AAC CCC GGC TTG GTC GCC AGG ATC ACC GAC AAG GGA CTG  144
  24  Leu Gly Ala Asn Pro Gly Leu Val Ala Arg Ile Thr Asp Lys Gly Leu   39

 145  CAG TAT GCG GCC CAG GAG GGG CTA TTG GCT CTG CAG AGT GAG CTG CTC  192
  40  Gln Tyr Ala Ala Gln Glu Gly Leu Leu Ala Leu Gln Ser Glu Leu Leu   55

 193  AGG ATC ACG CTG CCT GAC TTC ACC GGG GAC TTG AGG ATC CCC CAC GTC  240
  56  Arg Ile Thr Leu Pro Asp Phe Thr Gly Asp Leu Arg Ile Pro His Val   71

 241  GGC CGT GGG CGC TAT GAG TTC CAC AGC CTG AAC ATC CAC AGC TGT GAG  288
  72  Gly Arg Gly Arg Tyr Glu Phe His Ser Leu Asn Ile His Ser Cys Glu   87

 289  CTG CTT CAC TCT GCG CTG AGG CCT GTC CCT GGC CAG GGC CTG AGT CTC  336
  88  Leu Leu His Ser Ala Leu Arg Pro Val Pro Gly Gln Gly Leu Ser Leu  103

 337  AGC ATC TCC GAC TCC TCC ATC CGG GTC CAG GGC AGG TGG AAG GTG CGC  384
 104  Ser Ile Ser Asp Ser Ser Ile Arg Val Gln Gly Arg Trp Lys Val Arg  119

 385  AAG TCA TTC TTC AAA CTA CAG GGC TCC TTT GAT GTC AGT GTC AAG GGC  432
 120  Lys Ser Phe Phe Lys Leu Gln Gly Ser Phe Asp Val Ser Val Lys Gly  135

 433  ATC AGC ATT TCG GTC AAC CTC CTG TTG GGC AGC GAG TCC TCC GGG AGG  480
 136  Ile Ser Ile Ser Val Asn Leu Leu Leu Gly Ser Glu Ser Ser Gly Arg  151

 481  CCC ACA GTT ACT GCC TCC AGC TGC AGC AGT GAC ATC GCT GAC GTG GAG  528
 152  Pro Thr Val Thr Ala Ser Ser Cys Ser Ser Asp Ile Ala Asp Val Glu  167

 529  GTG GAC ATG TCG GGA GAC TTG GGG TGG CTG TTG AAC CTC TTC CAC AAC  576
 168  Val Asp Met Ser Gly Asp Phe Gly Trp Leu Leu Asn Leu Phe His Asn  183

 577  CAG ATT GAG TCC AAG TTC CAG AAA GTA CTG GAG AGC AGG ATT TGC GAA  624
 184  Gln Ile Glu Ser Lys Phe Gln Lys Val Leu Glu Ser Arg Ile Cys Glu  199

 625  ATG ATC CAG AAA TCG GTG TCC TCC GAT CTA CAG CCT TAT CTC CAA ACT  672
 200  Met Ile Gln Lys Ser Val Ser Ser Asp Leu Gln Pro Tyr Leu Gln Thr  215

 673  CTG CCA GTT ACA ACA GAG ATT GAC AGT TTC GCC GAC ATT GAT TAT AGC  720
 216  Leu Pro Val Thr Thr Glu Ile Asp Ser Phe Ala Asp Ile Asp Tyr Ser  231

 721  TTA GTG GAA GCC CCT CGG GCA ACA GCC CAG ATG CTG GAG GTG ATG TTT  768
 232  Leu Val Glu Ala Pro Arg Ala Thr Ala Gln Met Leu Glu Val Met Phe  247

 769  AAG GGT GAA ATC TTT CAT CGT AAC CAC CGT TCT CCA GTT ACC CTC CTT  816
 248  Lys Gly Glu Ile Phe His Arg Asn His Arg Ser Pro Val Thr Leu Leu  263

 817  GCT GCA GTC ATG AGC CTT CCT GAG GAA CAC AAC AAA ATG GTC TAC TTT  864
 264  Ala Ala Val Met Ser Leu Pro Glu Glu His Asn Lys Met Val Tyr Phe  279

 865  GCC ATC TCG GAT TAT GTC TTC AAC ACG GCC AGC CTG GTT TAT CAT GAG  912
 280  Ala Ile Ser Asp Tyr Val Phe Asn Thr Ala Ser Leu Val Tyr His Glu  295

 913  GAA GGA TAT CTG AAC TTC TCC ATC ACA GAT GAC ATG ATA CCG CCT GAC  960
 296  Glu Gly Tyr Leu Asn Phe Ser Ile Thr Asp Asp Met Ile Pro Pr  Asp  311

 961  TCT AAT ATC CGA CTG ACC ACC AAG TCC TTC CGA CCC TTC GTC CCA CGG 1008
 312  S r Asn Ile Arg Leu Thr Thr Lys Ser Phe Arg Pro Phe Val Pro Arg  327
```

FIGURE 4B

```
1009  TTA GCC AGG CTC TAC CCC AAC ATG AAC CTG GAA CTC CAG GGA TCA GTG   1056
 328  Leu Ala Arg Leu Tyr Pro Asn Met Asn Leu Glu Leu Gln Gly Ser Val    343

1057  CCC TCT GCT CCG CTC CTG AAC TTC AGC CCT GGG AAT CTG TCT GTG GAC   1104
 344  Pro Ser Ala Pro Leu Leu Asn Phe Ser Pro Gly Asn Leu Ser Val Asp    359

1105  CCC TAT ATG GAG ATA GAT GCC TTT GTG CTC CTG CCC AGC TCC AGC AAG   1152
 360  Pro Tyr Met Glu Ile Asp Ala Phe Val Leu Leu Pro Ser Ser Ser Lys    375

1153  GAG CCT GTC TTC CGG CTC AGT GTG GCC ACT AAT GTG TCC GCC ACC TTG   1200
 376  Glu Pro Val Phe Arg Leu Ser Val Ala Thr Asn Val Ser Ala Thr Leu    391

1201  ACC TTC AAT ACC AGC AAG ATC ACT GGG TTC CTG AAG CCA GGA AAG GTA   1248
 392  Thr Phe Asn Thr Ser Lys Ile Thr Gly Phe Leu Lys Pro Gly Lys Val    407

1249  AAA GTG GAA CTG AAA GAA TCC AAA GTT GGA CTA TTC AAT GCA GAG CTG   1296
 408  Lys Val Glu Leu Lys Glu Ser Lys Val Gly Leu Phe Asn Ala Glu Leu    423

1297  TTG GAA GCG CTC CTC AAC TAT TAC ATC CTT AAC ACC TTC TAC CCC AAG   1344
 424  Leu Glu Ala Leu Leu Asn Tyr Tyr Ile Leu Asn Thr Phe Tyr Pro Lys    439

1345  TTC AAT GAT AAG TTG GCC GAA GGC TTC CCC CTT CCT CTG CTG AAG CGT   1392
 440  Phe Asn Asp Lys Leu Ala Glu Gly Phe Pro Leu Pro Leu Leu Lys Arg    455

1393  GTT CAG CTC TAC GAC CTT GGG CTG CAG ATC CAT AAG GAC TTC CTG TTC   1440
 456  Val Gln Leu Tyr Asp Leu Gly Leu Gln Ile His Lys Asp Phe Leu Phe    471

1441  TTG GGT GCC AAT GTC CAA TAC ATG AGA GTT TGA GGA CAA GAA AGA TGA   1488
 472  Leu Gly Ala Asn Val Gln Tyr Met Arg Val ***                       482

1489  AGC TTG CTC GAG                                                  1500
           XhoI
```

FIGURE 5A

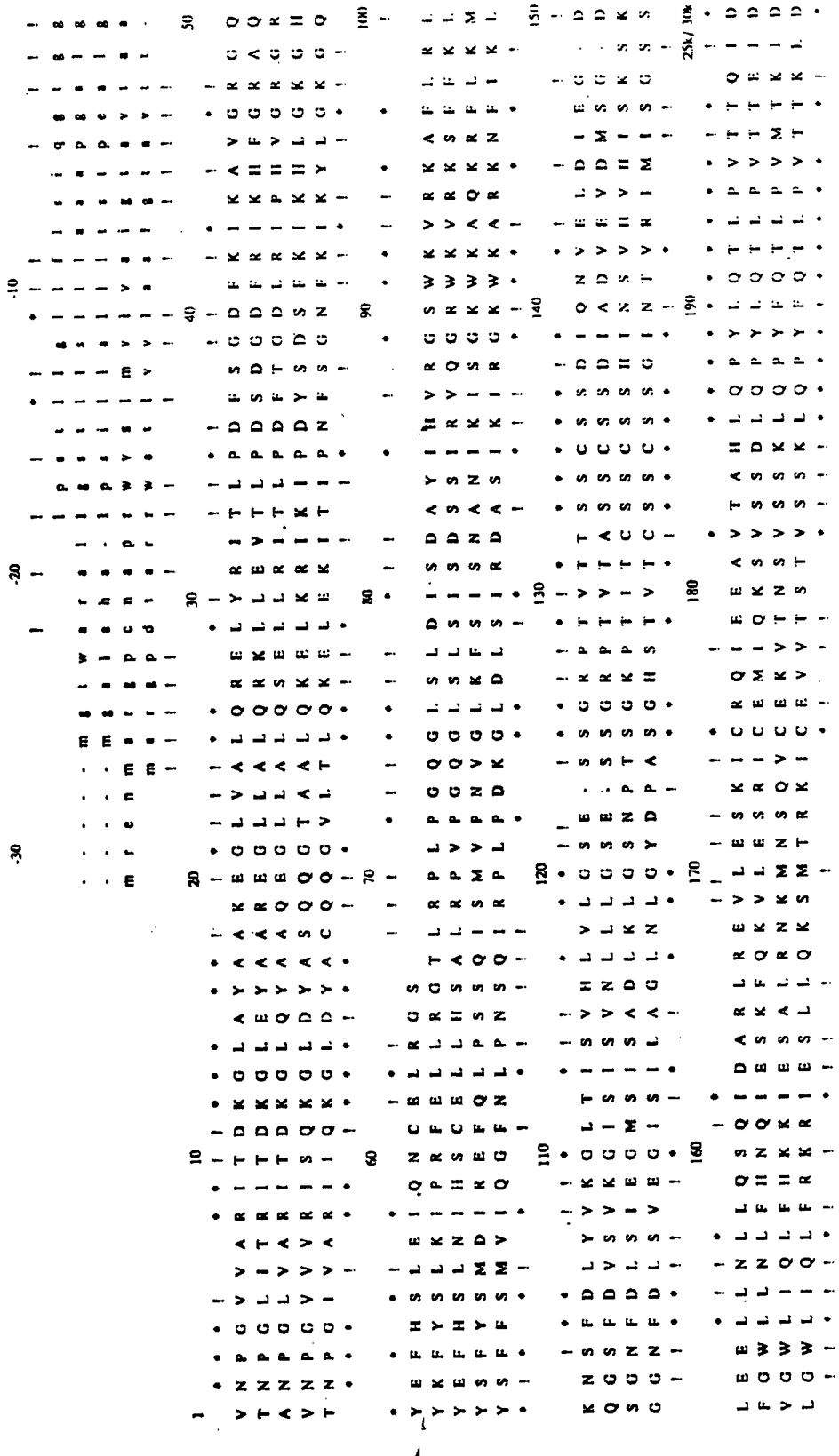


FIGURE 5B

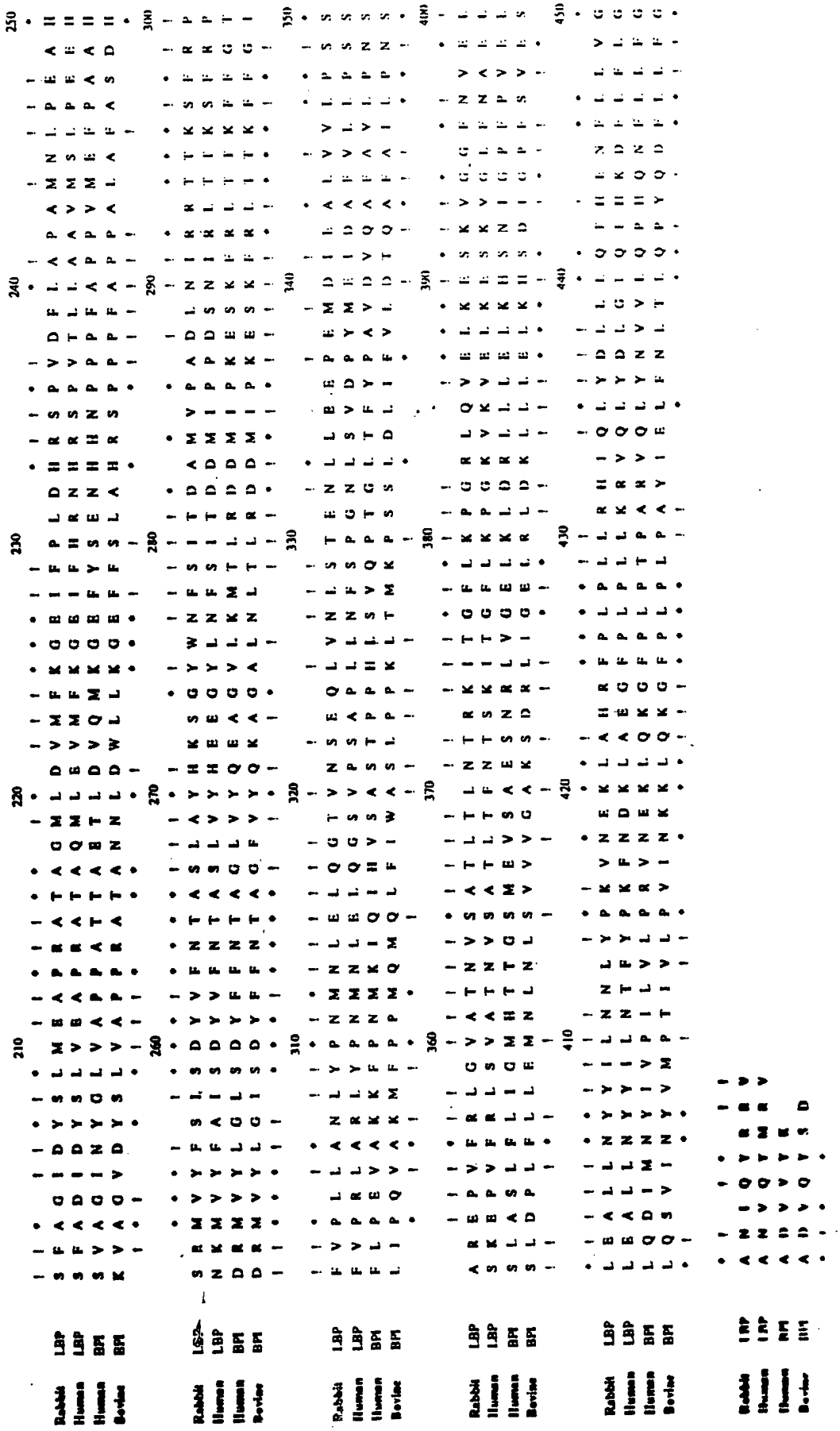


Figure 6

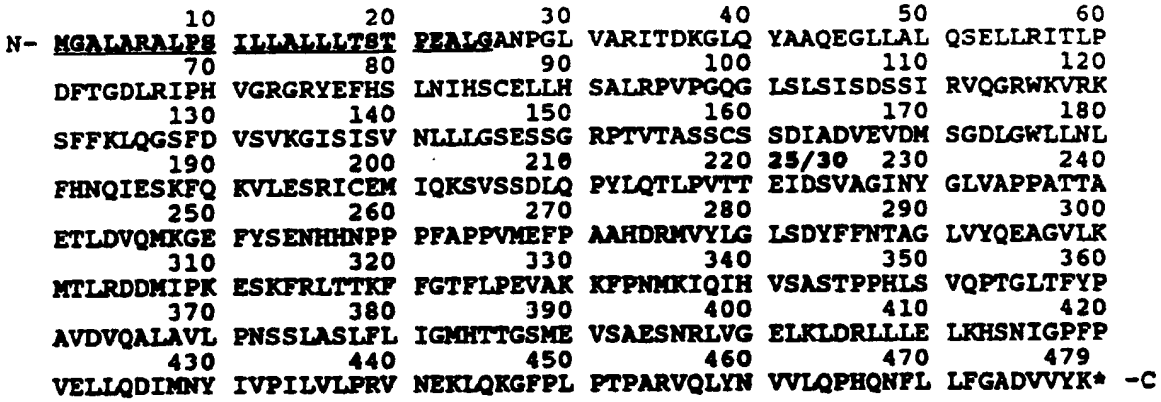

10 20 30 40 50 60
N- MGALARALPS ILLALLLTST PEALGANPGL VARITDKGLQ YAAQEGLLAL QSELLRITLP
70 80 90 100 110 120
DFTGDLRIPH VGRGRYEFHS LNIHSCELLH SALRPVPGQG LSLSISDSSI RVQGRWKVRK
130 140 150 160 170 180
SFFKLQGSFD VSVKGISISV NLLLGSESSG RPTVTASSCS SDIADVEVDM SGDLGWLLNL
190 200 210 220 25/30 230 240
FHNQIESKFQ KVLESRICEM IQKSVSSDLQ PYLQTLPVTT EIDSVAGINY GLVAPPATTA
250 260 270 280 290 300
ETLDVQMKGE FYSENHHNPP PFAPPVMEFP AAHDRMVYLG LSDYFFNTAG LVYQEAGVLK
310 320 330 340 350 360
MTLRDDMIPK ESKFRLTTKF FGTFLPEVAK KFPNMKIQIH VSASTPPHLS VQPTGLTFYP
370 380 390 400 410 420
AVDVQALAVL PNSSLASLFL IGMHTTGSME VSAESNRLVG ELKLDRLLLE LKHSNIGPFP
430 440 450 460 470 479
VELLQDIMNY IVPILVLPRV NEKLQKGFPL PTPARVQLYN VVLQPHQNFL LFGADVVYK* -C

FIGURE 7A

Human IgG1 cDNA

```
   1  CAC AAG ATC ATG AAA CAC CTG TGG TTC CTC CTC CTC TGG TGT CAG CTC    48
   1  His Lys Ile Met Lys His Leu Trp Phe Leu Leu Leu Trp Cys Gln Leu    16

  49  CCA GAT GTG AGG GTC CTG TCC CAG GTG CAG CTA CAG CAG TGG GGC GCA    96
  17  Pro Asp Val Arg Val Leu Ser Gln Val Gln Leu Gln Gln Trp Gly Ala    32

  97  GGA CTG GTG AAG CCT TCG GAG ACC CTG TCC CTC ACC TGC GCT GTC TTT   144
  33  Gly Leu Val Lys Pro Ser Glu Thr Leu Ser Leu Thr Cys Ala Val Phe    48

 145  GGT GGG TCC TTC AGT GGT TAC TAC TGG AGC TGG ATC CGC CAG CCC CCA   192
  49  Gly Gly Ser Phe Ser Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro    64

 193  GGA AGG GGA CTG GAG TGG ATT GGA GAA ATC AAT CAT AGT GGA AGC ACC   240
  65  Gly Arg Gly Leu Glu Trp Ile Gly Glu Ile Asn His Ser Gly Ser Thr    80

 241  AAT TAC AAA ACG TCC CTC AAG AGT CGA GTC ACC ATA TCT TTA GAC ACG   288
  81  Asn Tyr Lys Thr Ser Leu Lys Ser Arg Val Thr Ile Ser Leu Asp Thr    96

 289  TCC AAG AAC CTG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCG GAC   336
  97  Ser Lys Asn Leu Phe Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp   112

 337  ACG GCT GTG TAT TAC TGT GCG AGG GGC CTC CTC CGG GGG GGC TGG AAC   384
 113  Thr Ala Val Tyr Tyr Cys Ala Arg Gly Leu Leu Arg Gly Gly Trp Asn   128

 385  GAC GTG GAC TAC TAC TAT GGT ATG GAC GTC TGG GGC CAA GGG ACC ACG   432
 129  Asp Val Asp Tyr Tyr Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr   144

 433  GTC ACC GTC TCC TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG   480
 145  Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu   160
                  ^^^site 1
 481  GCA CCC TCC TCC AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC   528
 161  Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys   176

 529  CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA   576
 177  Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser   192

 577  GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC   624
 193  Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser   208

 625  TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC   672
 209  Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser   224

 673  TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC   720
 225  Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn   240

 721  ACC AAG GTG GAC AAG AAA GCA GAG CCC AAA TCT TGT GAC AAA ACT CAC   768
 241  Thr Lys Val Asp Lys Lys Ala Glu Pro Lys Ser Cys Asp Lys Thr His   256
                                      ^^^site 2
 769  ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC   816
 257  Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val   272

 817  TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC   864
 273  Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr   288

 865  CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG   912
 289  Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu   304

 913  GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG   960
 305  Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys   320

 961  ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGG GTG GTC AGC  1008
 321  Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser   336

1009  GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG  1056
```

FIGURE 7B

337	Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys	352
1057	TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC	1104
353	Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile	368
1105	TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC	1152
369	Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro	384
1153	CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG	1200
385	Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu	400
1201	GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT	1248
401	Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn	416
1249	GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC	1296
417	Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser	432
1297	GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG	1344
433	Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg	448
1345	TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG	1392
449	Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu	464
1393	CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA	1437
465	His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys	479

Figure 8

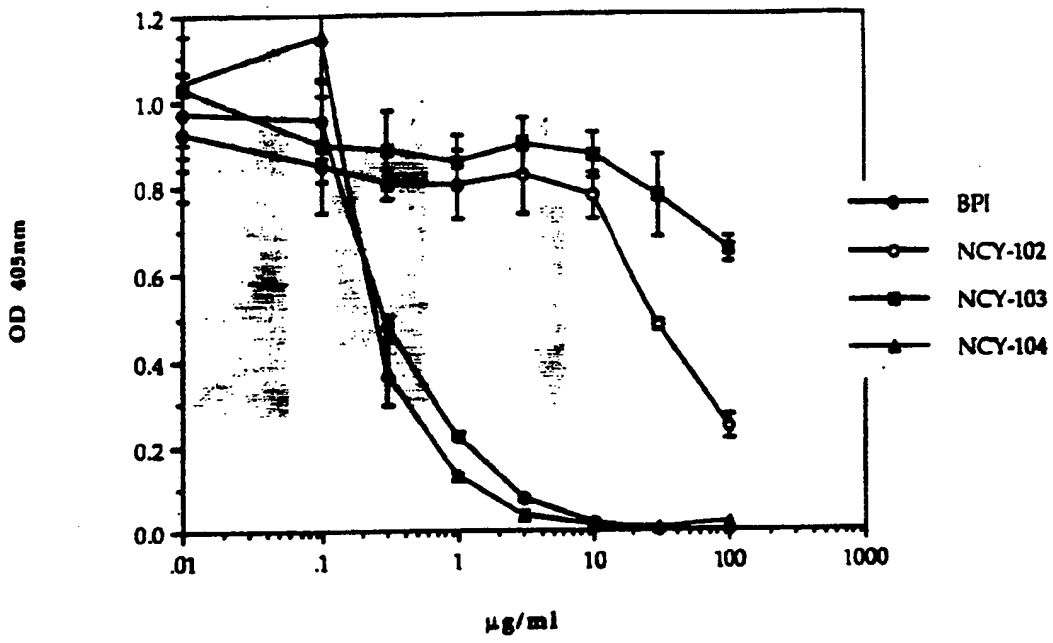

OD 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
.01
.1
1
10
100
1000
µg/ml
BPI
NCY-102
NCY-103
NCY-104

Figure 9

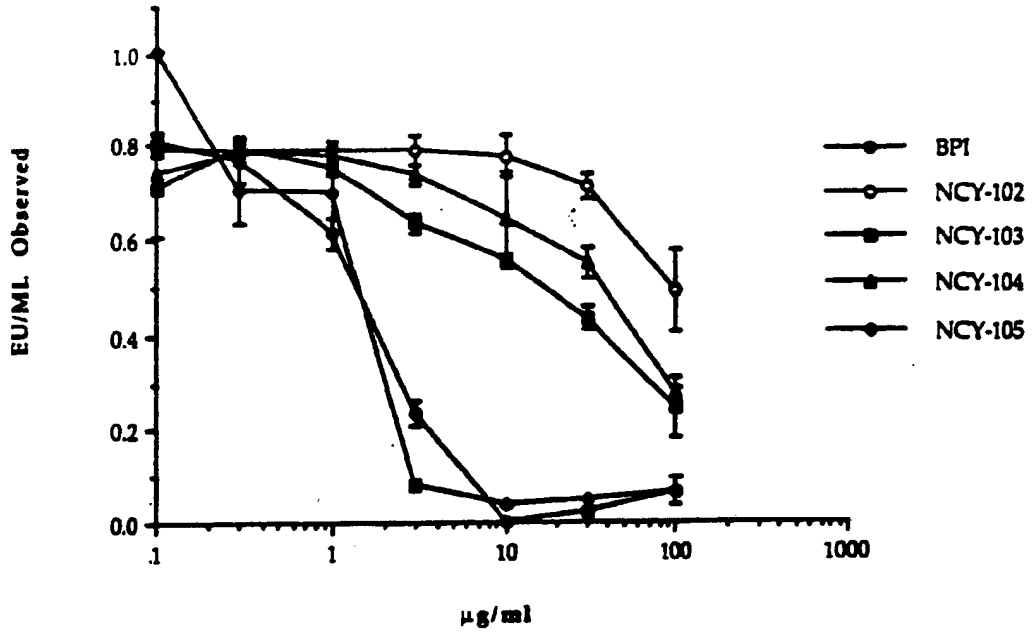

EU/ML Observed
1.0
0.8
0.6
0.4
0.2
0.0
.1
1
10
100
1000
µg/ml
BPI
NCY-102
NCY-103
NCY-104
NCY-105

Figure 10

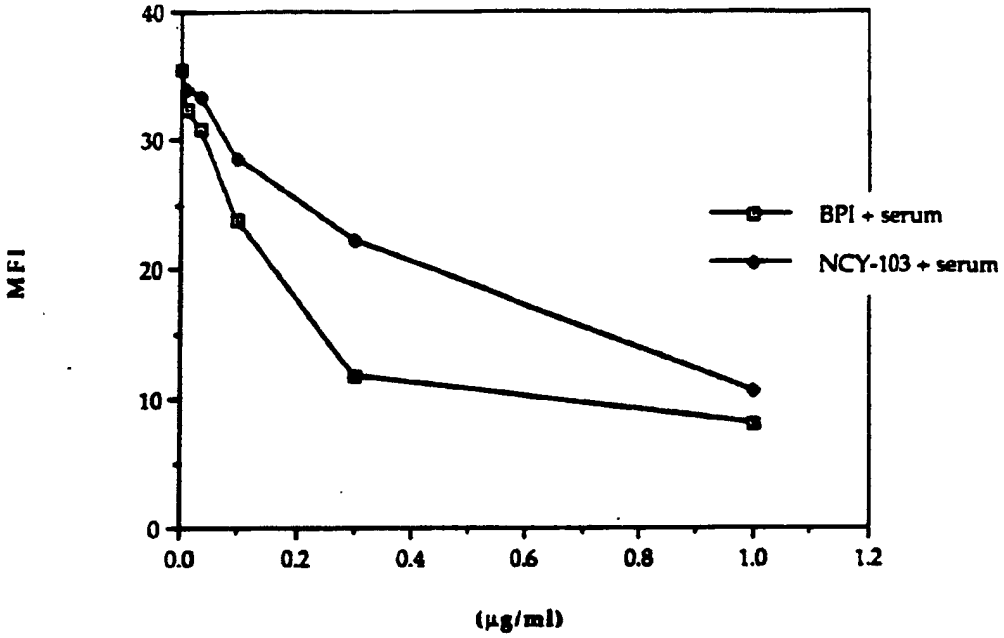


Figure 11

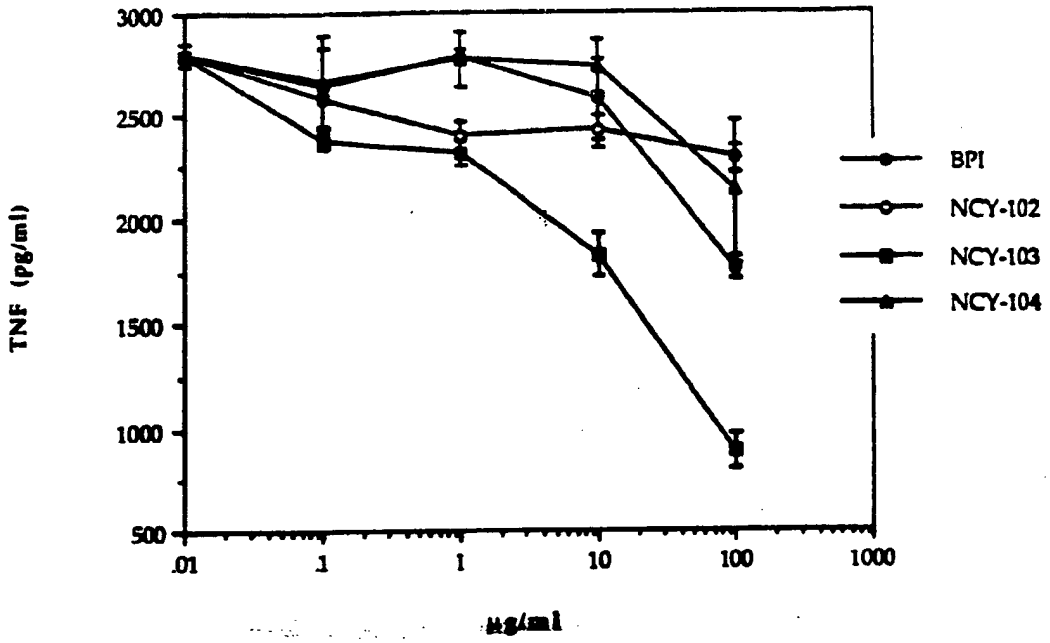

3000
2500
2000
1500
1000
500
TNF (pg/ml)
.01
.1
1
10
100
1000
µg/ml
BPI
NCY-102
NCY-103
NCY-104

Figure 12

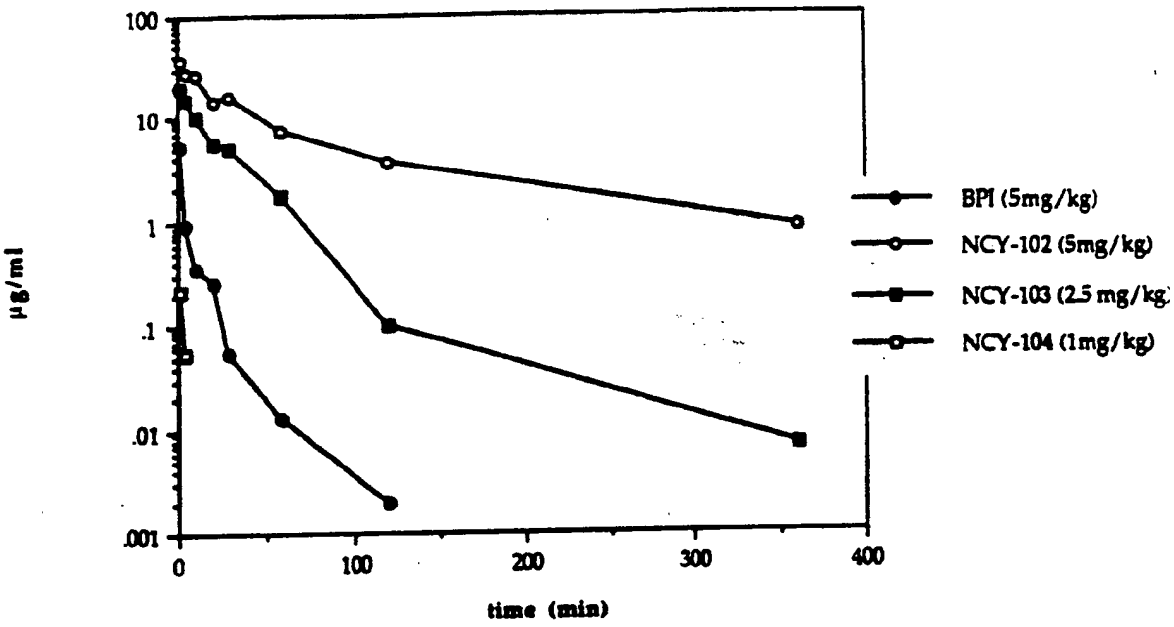

100
10
1
.1
.01
.001
µg/ml
0
100
200
300
400
time (min)
BPI (5mg/kg)
NCY-102 (5mg/kg)
NCY-103 (2.5 mg/kg)
NCY-104 (1mg/kg)

Figure 13

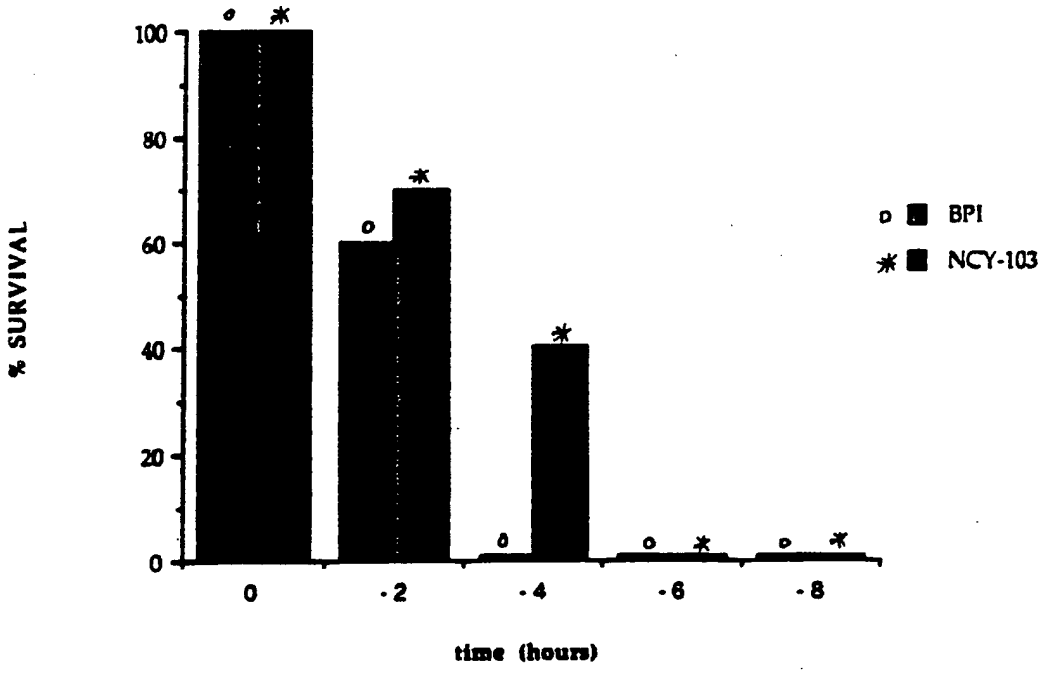


FIGURE 14

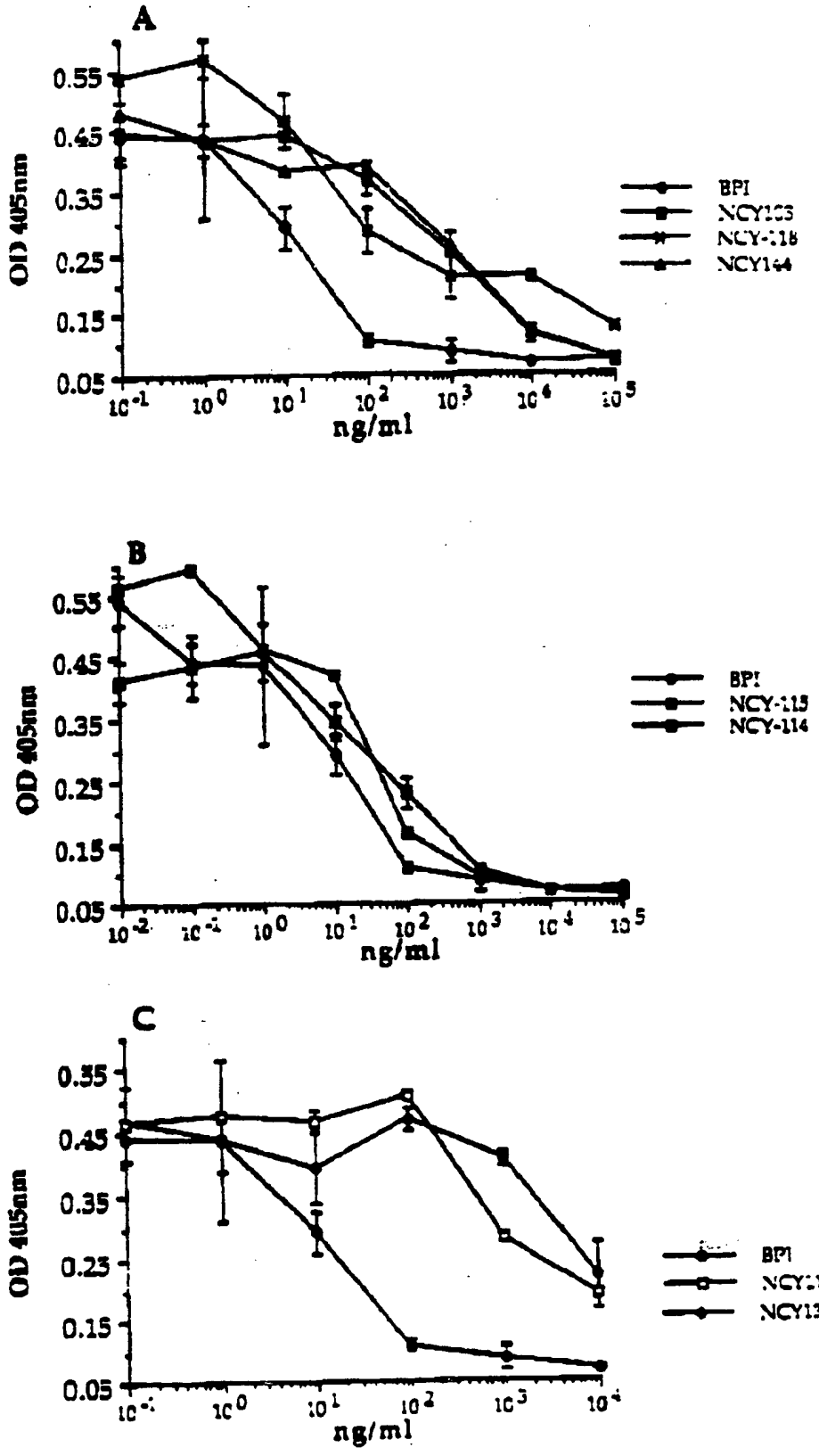
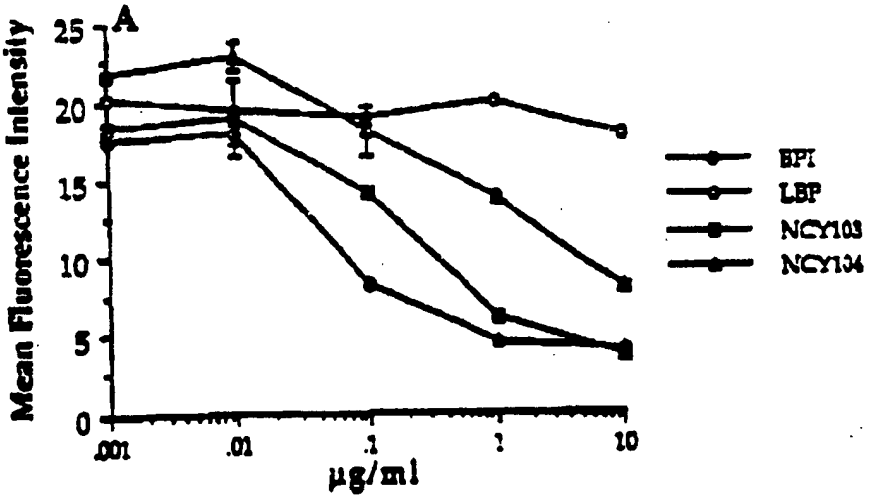

A
OD 405nm
0.55
0.45
0.35
0.25
0.15
0.05
ng/ml
BPI
NCY103
NCY-118
NCY144
B
OD 405nm
0.55
0.45
0.35
0.25
0.15
0.05
ng/ml
BPI
NCY-115
NCY-114
C
OD 405nm
0.55
0.45
0.35
0.25
0.15
0.05
ng/ml
BPI
NCY117
NCY139

FIGURE 15


A
Mean Fluorescence Intensity
25
20
15
10
5
0
.001
.01
.1
1
10
µg/ml
BPI
LBP
NCY103
NCY104

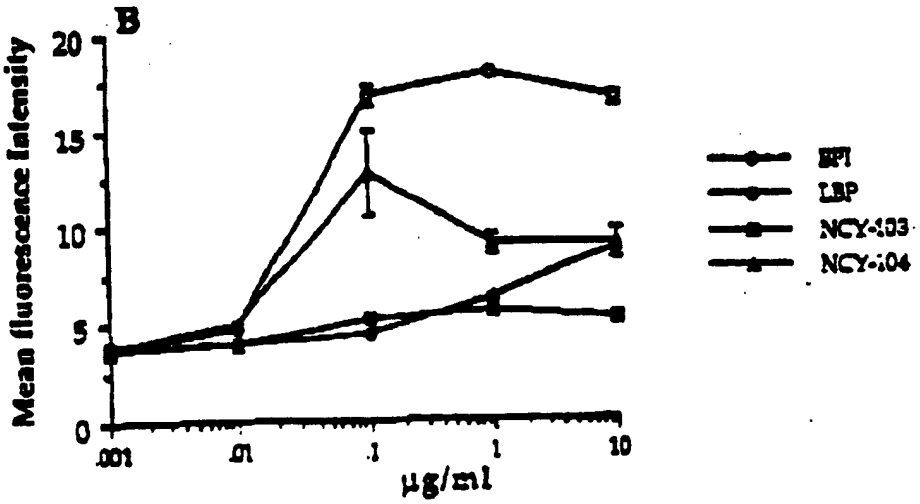

B
Mean fluorescence Intensity
20
15
10
5
0
.001
.01
.1
1
10
µg/ml
BPI
LBP
NCY-103
NCY-104

FIGURE 16

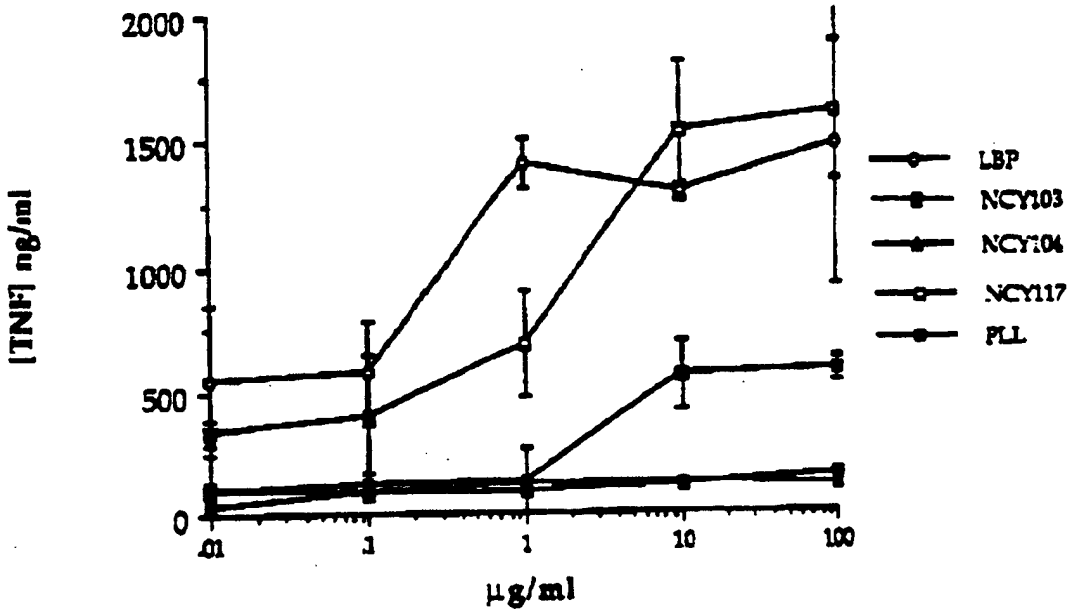

2000
1500
1000
500
0
[TNF] ng/ml
.01
.1
1
10
100
µg/ml
LBP
NCY103
NCY104
NCY117
PLL

FIGURE 17

LPS-Mediated TNF Production in THP-1 Cells Cultured Without Serum

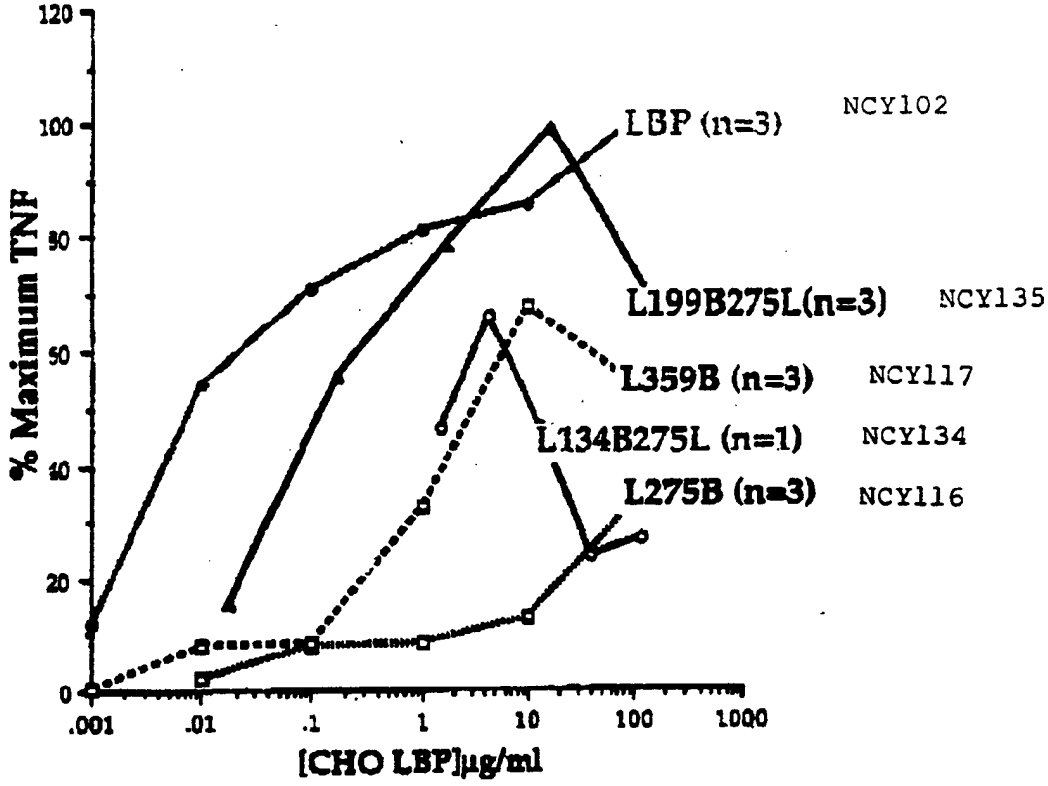
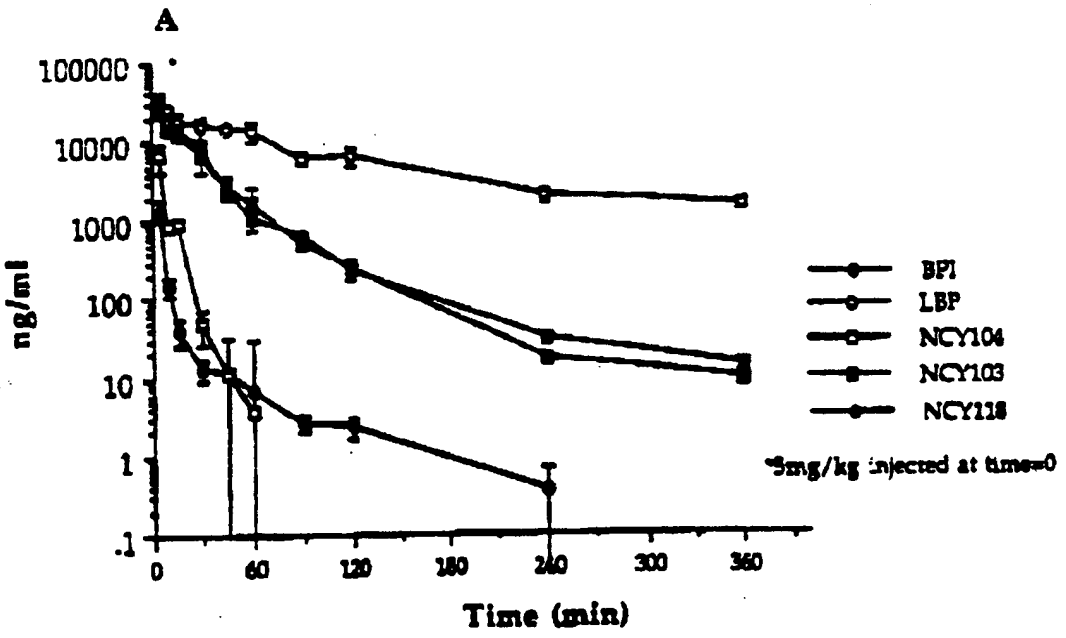


FIGURE 18A


A
100000
10000
1000
100
10
1
.1
ng/ml
0
60
120
180
240
300
360
Time (min)
BPI
LBP
NCY104
NCY103
NCY118
*5mg/kg injected at time=0

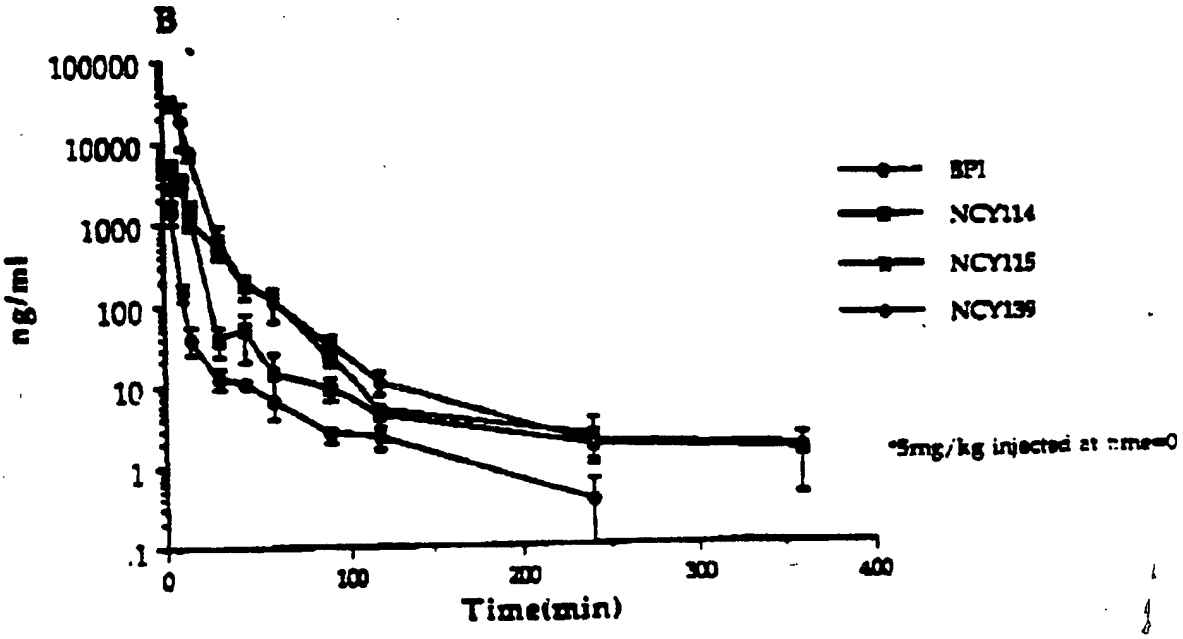
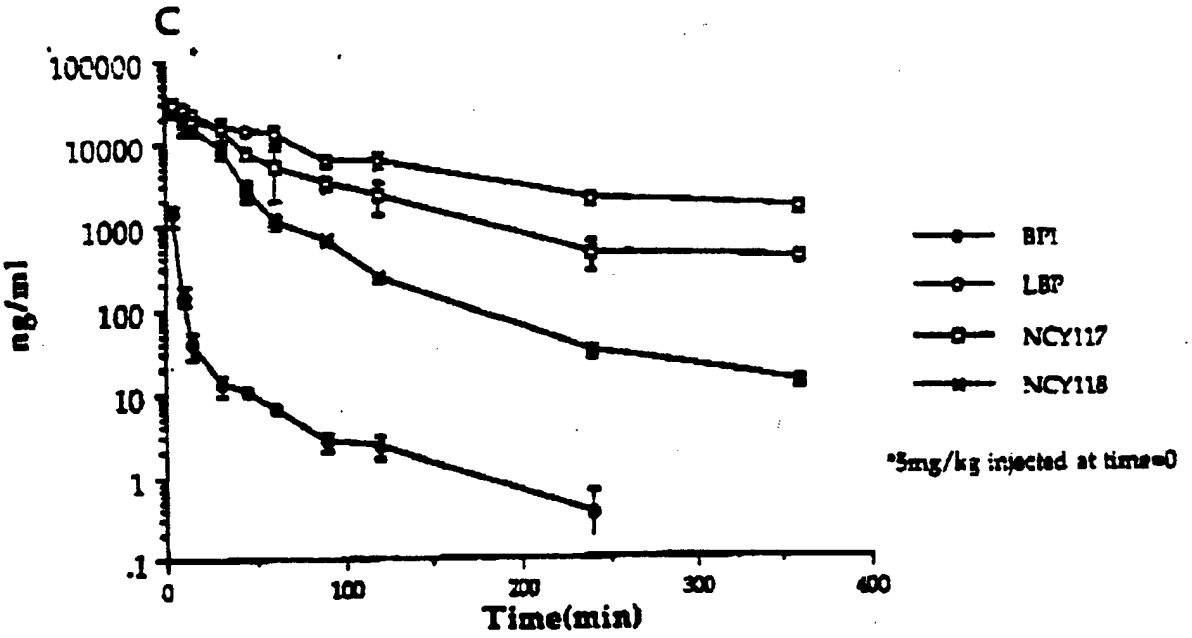
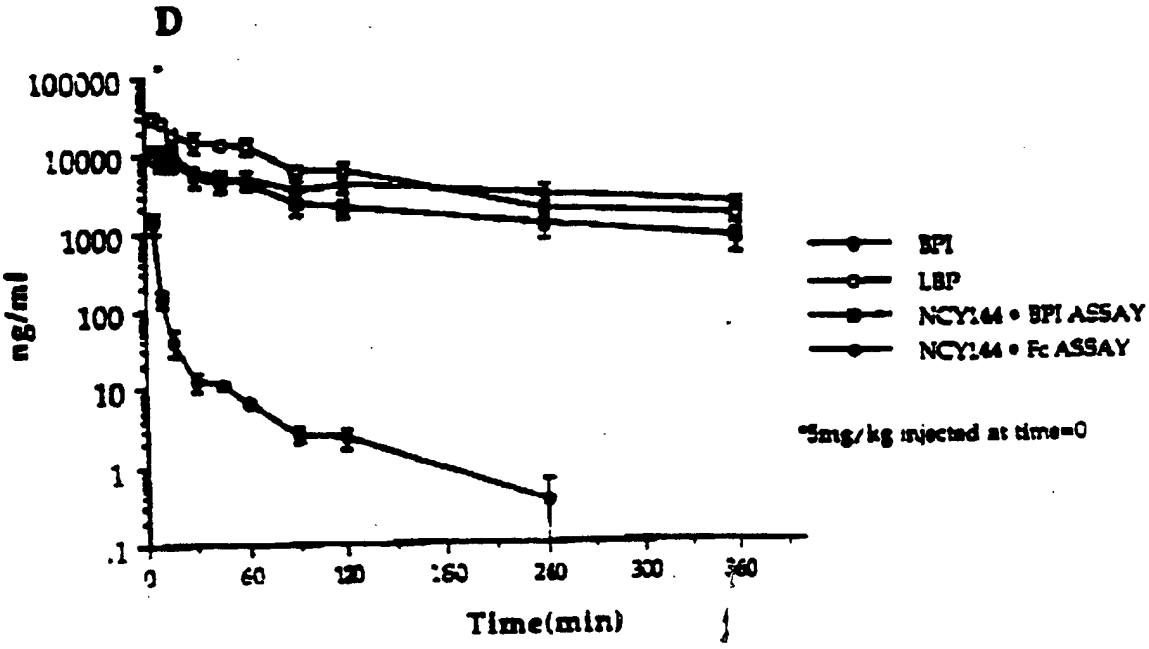

B
100000
10000
1000
100
10
1
.1
ng/ml
0
100
200
300
400
Time(min)
BPI
NCY114
NCY115
NCY139
*5mg/kg injected at time=0

FIGURE 18B


C
100000
10000
1000
100
10
1
.1
ng/ml
0
100
200
300
400
Time(min)
BPI
LBP
NCY117
NCY118
*5mg/kg injected at time=0


D
100000
10000
1000
100
10
1
.1
ng/ml
0
60
120
180
240
300
360
Time(min)
BPI
LBP
NCY144 • BPI ASSAY
NCY144 • Fc ASSAY
*5mg/kg injected at time=0

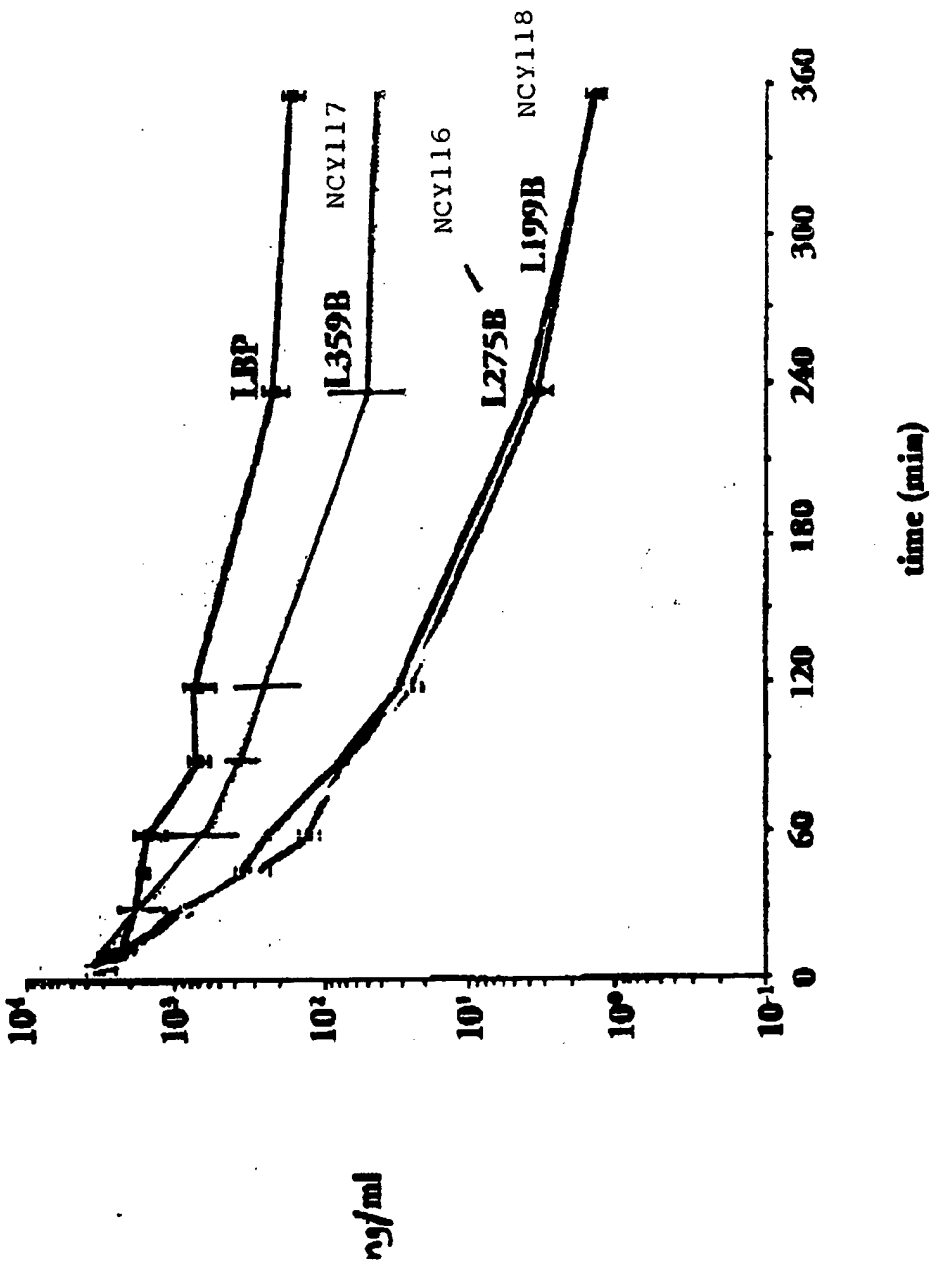

LBP
L359B
NCY117
NCY116
L275B
L199B
NCY118
ng/ml
time (min)
0
60
120
180
240
300
360

FIGURE 18C

FIGURE 18D

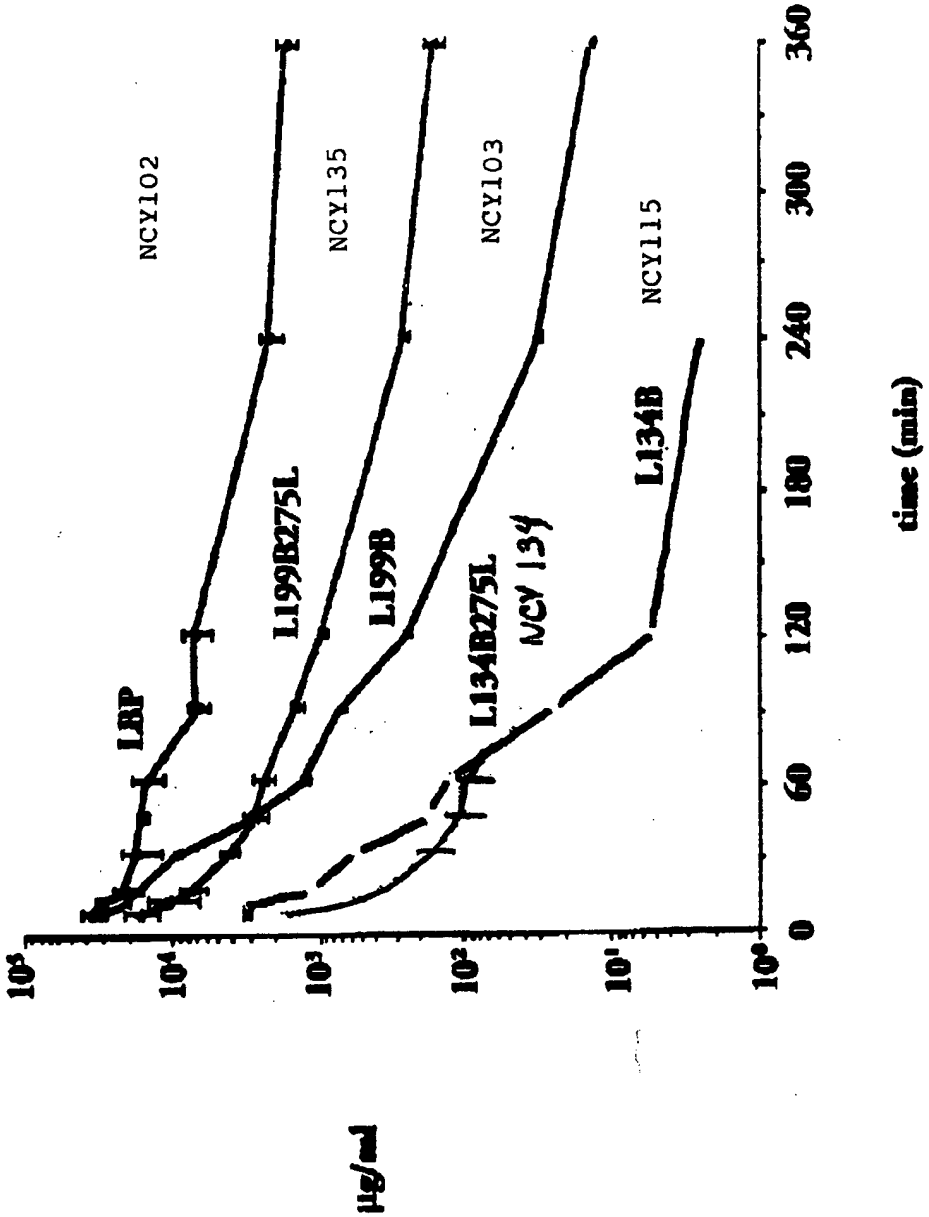

NCY102
NCY135
NCY103
NCY115
LBP
L199B275L
L199B
L134B275L
NCY 134
L134B
10^5
10^4
10^3
10^2
10^1
10^0
0
60
120
180
240
300
360
time (min)
µg/ml

FIGURE 18E

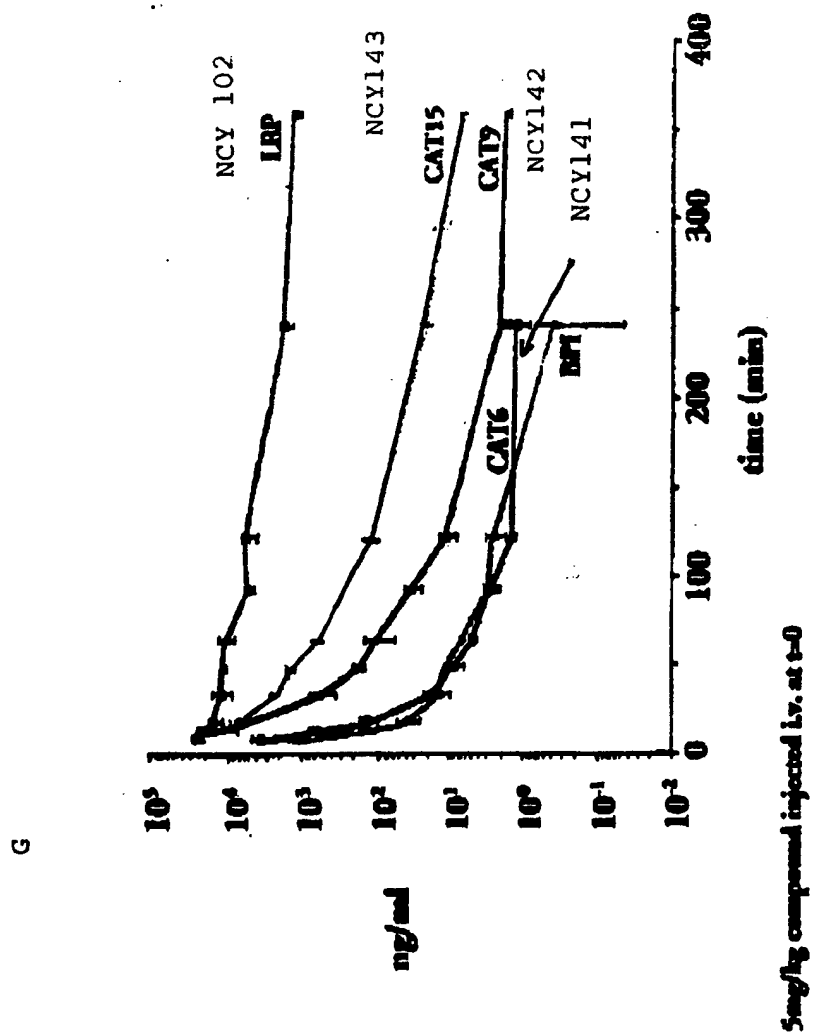

NCY 102
LBP
NCY143
CAT15
CAT9
NCY142
NCY141
CAT6
BPI
ng/ml
time (min)
5mg/kg compound injected i.v. at t=0

FIGURE 18F

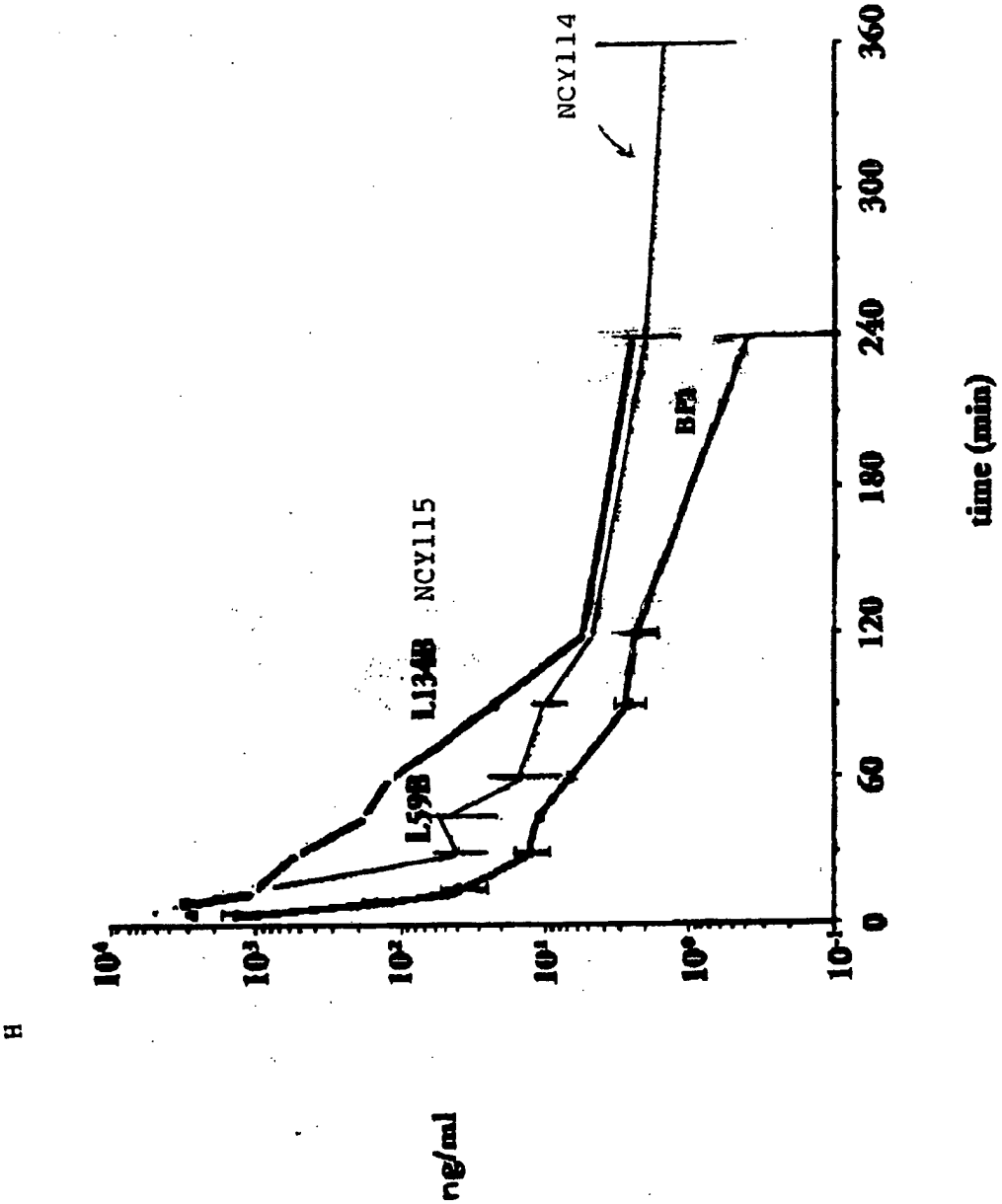

NCY114
NCY115
L134B
L59B
BPA
10^4
10^3
10^2
10^1
10^0
10^{-1}
0
60
120
180
240
300
360
time (min)
ng/ml
H

FIGURE 19

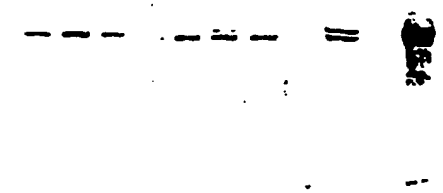
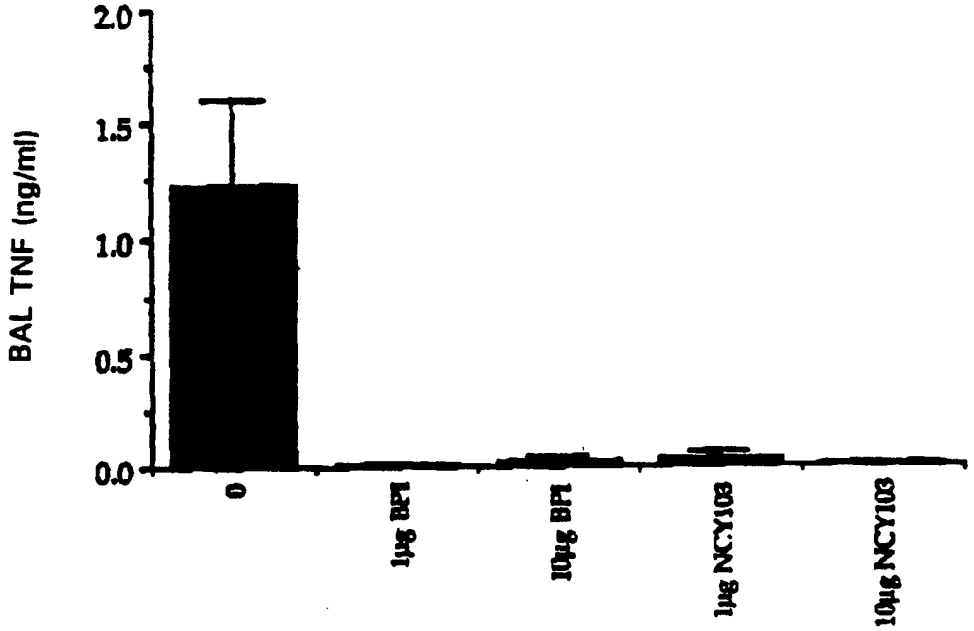


FIGURE 20


BAL TNF (ng/ml)
2.0
1.5
1.0
0.5
0.0
0
1μg BPI
10μg BPI
1μg NCY103
10μg NCY103

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04709

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C07H 15/12; C12P 21/06; A61K 39/00; C07K 3/00; A61K 37/00
US CL : 536/27; 435/69.1, 69.3; 424/88; 530/350; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/27; 435/69.1, 69.3; 424/88; 530/350; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemical and Biophysical Research Communications, Volume 179, No.1, issued 30 August 1991, Larrick et al "Complementary DNA Sequence of Rabbit CAP18--A Unique Lipopolysaccharide Binding Protein", pages 170-175, see the Abstract, pages 170 and 171.	3, 10-15
Y	Journal of Experimental Medicine, Volume 174, issued September 1991, Ooi et al, "Endotoxin-neutralizing Properties of the 25 kD N-Terminal fragment and a Newly Isolated 30 kD C-Terminal Fragment of the 55-60 kD Bactericidal/Permeability-increasing Protein of Human Neutrophils", pages 649-655, see page 649.	1, 2, 4-9, 16-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 AUGUST 1994	AUG 11 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer H.F. Sidberry Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04709

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Biological Chemistry, Vol. 264, No. 16, issued 05 June 1989, Gray et al, "Cloning of the cDNA of a Human Neutrophil Bactericidal Protein", pages 9505-9509, see pages 9505, 9508, 9509.	1,2, 5-9

INTERNATIONAL SEARCH REPORT

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B FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

CAS, MEDLINE, TOXLINE, DIALOG
search terms: bactericidal/permeability increasing protein,
chimera?, variant?